DIALOG search notes 3/04 BP 1/2

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File 155:MEDLINE(R)  1966-2004/Mar W1
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*File 155: Medline has been reloaded. Accession numbers
have changed. Please see HELP NEWS 154 for details.

      Set  Items  Description
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?e botulinum

Ref    Items    RT  Index-term
E1         1        BOTULINOWYCH
E2         1        BOTULINUE
E3      7206       *BOTULINUM
E4      1650        BOTULINUM //CLOSTRIDIUM (CLOSTRIDIUM BOTULINUM)
E5       186     2  BOTULINUM ANTITOXIN
E6        15        BOTULINUM ANTITOXIN --ADMINISTRATION AND DOSAG
E7         6        BOTULINUM ANTITOXIN --ADVERSE EFFECTS --AE
E8        14        BOTULINUM ANTITOXIN --ANALYSIS --AN
E9         1        BOTULINUM ANTITOXIN --BIOSYNTHESIS --BI
E10        1        BOTULINUM ANTITOXIN --BLOOD --BL
E11        1        BOTULINUM ANTITOXIN --CLASSIFICATION --CL
E12        1        BOTULINUM ANTITOXIN --DIAGNOSTIC USE --DU

          Enter P or PAGE for more
?p

Ref    Items    RT  Index-term
E13        1        BOTULINUM ANTITOXIN --ECONOMICS --EC
E14        1        BOTULINUM ANTITOXIN --HISTORY --HI
E15        7        BOTULINUM ANTITOXIN --IMMUNOLOGY --IM
E16        5        BOTULINUM ANTITOXIN --ISOLATION AND PURIFICATI
E17        1        BOTULINUM ANTITOXIN --METABOLISM --ME
E18       11        BOTULINUM ANTITOXIN --PHARMACOLOGY --PD
E19        1        BOTULINUM ANTITOXIN --STANDARDS --ST
E20        3        BOTULINUM ANTITOXIN --SUPPLY AND DISTRIBUTION
E21       90        BOTULINUM ANTITOXIN --THERAPEUTIC USE --TU
E22        1        BOTULINUM ANTITOXIN --TOXICITY --TO
E23     1322     4  BOTULINUM TOXIN TYPE A
E24      477        BOTULINUM TOXIN TYPE A --ADMINISTRATION AND DO

          Enter P or PAGE for more
?p

Ref    Items  Index-term
E25      196  BOTULINUM TOXIN TYPE A --ADVERSE EFFECTS --AE
E26       12  BOTULINUM TOXIN TYPE A --ANALYSIS --AN
E27       21  BOTULINUM TOXIN TYPE A --ANTAGONISTS AND INHIB
E28        8  BOTULINUM TOXIN TYPE A --BIOSYNTHESIS --BI
E29        3  BOTULINUM TOXIN TYPE A --BLOOD --BL
E30        2  BOTULINUM TOXIN TYPE A --CHEMICAL SYNTHESIS --
E31       42  BOTULINUM TOXIN TYPE A --CHEMISTRY --CH
E32        1  BOTULINUM TOXIN TYPE A --CLASSIFICATION --CL
E33       10  BOTULINUM TOXIN TYPE A --CONTRAINDICATIONS --C
E34       12  BOTULINUM TOXIN TYPE A --ECONOMICS --EC
E35       22  BOTULINUM TOXIN TYPE A --GENETICS --GE
E36       10  BOTULINUM TOXIN TYPE A --HISTORY --HI

          Enter P or PAGE for more
?p

Ref    Items  Index-term
E37       45  BOTULINUM TOXIN TYPE A --IMMUNOLOGY --IM
E38       19  BOTULINUM TOXIN TYPE A --ISOLATION AND PURIFIC
E39       43  BOTULINUM TOXIN TYPE A --METABOLISM --ME
E40       17  BOTULINUM TOXIN TYPE A --PHARMACOKINETICS --PK
E41      198  BOTULINUM TOXIN TYPE A --PHARMACOLOGY --PD
E42        5  BOTULINUM TOXIN TYPE A --POISONING --PO
E43        3  BOTULINUM TOXIN TYPE A --STANDARDS --ST
E44        1  BOTULINUM TOXIN TYPE A --SUPPLY AND DISTRIBUTI
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E45     731  BOTULINUM TOXIN TYPE A --THERAPEUTIC USE --TU
E46      30  BOTULINUM TOXIN TYPE A --TOXICITY --TO
E47       1  BOTULINUM TOXIN TYPE A --URINE --UR
E48     151  BOTULINUM TOXIN TYPE B

          Enter P or PAGE for more
?s e37
      S1      45  'BOTULINUM TOXIN TYPE A --IMMUNOLOGY --IM'
?p

Ref   Items  Index-term
E49      96  BOTULINUM TOXIN TYPE C
E50      22  BOTULINUM TOXIN TYPE D
?s e48
      S2     151  'BOTULINUM TOXIN TYPE B'
?ds

Set     Items   Description
S1         45   'BOTULINUM TOXIN TYPE A --IMMUNOLOGY --IM'
S2        151   'BOTULINUM TOXIN TYPE B'
?s s2 and (carbox? or cterminal or (c(n)termin?))
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          109885  CARBOX?
               6  CTERMINAL
          808650  C
          353587  TERMIN?
           52468  C(N)TERMIN?
      S3      14  S2 AND (CARBOX? OR CTERMINAL OR (C(N)TERMIN?))
?s s2 and epitop?
             151  S2
           75778  EPITOP?
      S4       0  S2 AND EPITOP?
?s s2 and map?
             151  S2
          240265  MAP?
      S5       3  S2 AND MAP?
?t s5/9/all

 5/9/1
DIALOG(R)File 155:MEDLINE(R)
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14067626 PMID: 9767710

Characterization of the genes encoding the botulinum neurotoxin complex in a strain of Clostridium botulinum producing type B and F neurotoxins.

Santos-Buelga J A; Collins M D; East A K

Department of Microbiology, Institute of Food Research, Earley Gate, Whiteknights Road, Reading, RG6 6BZ, UK.

Current microbiology (UNITED STATES) Nov 1998, 37 (5) p312-8, ISSN 0343-8651 Journal Code: 7808448

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

The organization of the clusters of genes encoding proteins of the botulinum neurotoxin (BoNT) progenitor complex was elucidated in a strain of Clostridium botulinum producing type B and F neurotoxins. With PCR and sequencing strategies, the type B BoNT-gene cluster was found to be composed of genes encoding BoNT/B, nontoxic nonhemagglutinin component (NTNH), P-21, and the hemagglutinins HA-33, HA-17, and HA-70, whereas the type F BoNT-gene cluster has genes encoding BoNT/F, NTNH, P-47, and P-21. Comparative sequence analysis showed that BoNT/F in type BF strain 3281 shares highest homology with BoNT/F of non-proteolytic (group II) C. botulinum whereas NTNH and P-21 in the type F cluster of strain 3281 are more similar to the corresponding proteins in proteolytic (group I) type F C. botulinum. These findings indicate diverse evolutionary origins for genes encoding BoNT/F and its associated non-toxic proteins, although the genes are contiguous. By contrast, sequence comparisons indicate that genes

encoding BoNT/B and associated non-toxic proteins in strain 3281 possess a similar evolutionary origin. It was demonstrated that the genes present in the BoNT/B gene cluster of this type BF strain show exceptionally high homology with the equivalent genes in the silent BoNT/B gene cluster of C. botulinum type A(B), possibly indicating their common ancestry.

Tags: Human; Support, Non-U.S. Gov't

Descriptors: *Botulinum Toxins--genetics--GE; *Clostridium botulinum --genetics--GE; *Genes, Bacterial; *Multigene Family; Base Sequence; Botulinum Toxins--biosynthesis--BI; Chromosome **Mapping** ; Cloning, Molecular; Clostridium botulinum--metabolism--ME; Electrophoresis, Agar Gel ; Molecular Sequence Data; Polymerase Chain Reaction--methods--MT; Polymorphism, Restriction Fragment Length; Sequence Analysis, DNA; Sequence Homology, Amino Acid

Molecular Sequence Databank No.: GENBANK/Y13630; GENBANK/Y13631

CAS Registry No.: 0 (Botulinum Toxins); 0 (botulinum toxin type B); 0 (botulinum toxin type F)

Record Date Created: 19981202

Record Date Completed: 19981202

5/9/2

DIALOG(R)File 155:MEDLINE(R)

13979348 PMID: 9678262

Cloning and characterization of the upstream region of Clostridium botulinum type B neurotoxin gene.

Yang G H; Rhee S D; Jung H H; Jhee O H; Yang K H

Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Yusong, Taejon, Korea.

Biochemistry and molecular biology international (AUSTRALIA) Jun 1998, 45 (2) p401-7, ISSN 1039-9712 Journal Code: 9306673

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

The upstream region of the gene coding for Clostridium botulinum type B (BoNT/B) neurotoxin was cloned and sequenced. There were two open reading frames, which were identified as a nontoxic-nonhemagglutinin component (ntnh/B) and a 22 kDa adjacent open reading frame (orf22/B). Deduced primary structure of ntnh/B showed that it was composed of 1,197 amino acid residues. Pairwise comparisons of the ntnh/B component with other botulinum toxin types showed high degree of homology to ntnh/A (82% identity). Northern blot analysis revealed that toxin gene could be transcribed alone or co-transcribed with the ntnh gene. The orf22/B gene encoding for 178 amino acids (M.W. 21.6 kDa) was located between the 33 kDa hemagglutinin gene and the ntnh gene. Orf22/B also showed high degree of homology to orf22/A (98.9% identity). These results suggested that the upstream region of the BoNT/B gene (containing the ntnh/B and orf22/B genes) might be evolutionarily closely related to the counterparts of the BoNT/A.

Tags: Support, Non-U.S. Gov't

Descriptors: *Botulinum Toxins--genetics--GE; *Clostridium botulinum --genetics--GE; Amino Acid Sequence; Bacterial Proteins--chemistry--CH; Bacterial Proteins--genetics--GE; Base Sequence; Botulinum Toxins --chemistry--CH; Chromosome **Mapping** ; Cloning, Molecular; Genes, Bacterial ; Molecular Sequence Data; Molecular Weight; Open Reading Frames; Sequence Homology, Amino Acid; Transcription, Genetic

Molecular Sequence Databank No.: GENBANK/U63808

CAS Registry No.: 0 (Bacterial Proteins); 0 (Botulinum Toxins); 0 (NTNH protein, Clostridium botulinum); 0 (botulinum toxin type B)

Record Date Created: 19980925

Record Date Completed: 19980925

5/9/3

DIALOG(R)File 155:MEDLINE(R)

12030523 PMID: 12350104

Characterisation of botulinum toxins type A and B, by matrix-assisted laser desorption ionisation and electrospray mass spectrometry.

van Baar Ben L M; Hulst Albert G; de Jong Ad L; Wils Eric R J

TNO Prins Maurits Laboratory, Division Chemical and Biological Protection, Rijswijk, The Netherlands. baar@pml.tno.nl

Journal of chromatography. A (Netherlands) Sep 13 2002, 970 (1-2) p95-115, Journal Code: 9318488

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

A method earlier developed for the mass spectrometric (MS) identification of tetanus toxin (TTx) was applied to botulinum toxins type A and B (BTxA and BTxB). Botulinum toxins are extremely neurotoxic bacterial toxins, likely to be used as biological warfare agent. Biologically active BTxA and BTxB are comprised of a protein complex of the respective neurotoxins with specific haemagglutinins (HAs) and non-toxic non-haemagglutinins (NTNHs). These protein complexes are also observed in mass spectrometric identification. The particular BTxA complex, from Clostridium botulinum strain 62A, almost completely matched database data derived from genetic sequences known for this strain. Although no such database information was available for BTxB, from C. botulinum strain okra, all protein sequences from the complex except that of HA-70 were found to match proteins known from other type B strains. It was found that matrix-assisted laser desorption ionisation MS provides provisional identification from trypsin digest peptide **maps** and that liquid chromatography electrospray (tandem) mass spectrometry affords unequivocal identification from amino acid sequence information of digest peptides obtained in trypsin or pepsin digestion.

Tags: Support, Non-U.S. Gov't

Descriptors: *Botulinum Toxin Type A--chemistry--CH; *Botulinum Toxins --chemistry--CH; *Spectrometry, Mass, Electrospray Ionization--methods--MT; *Spectrometry, Mass, Matrix-Assisted Laser Desorption-Ionization--methods --MT; Amino Acid Sequence; Molecular Sequence Data; Pepsin A--chemistry--CH ; Sequence Homology, Amino Acid; Trypsin--chemistry--CH

CAS Registry No.: 0 (Botulinum Toxin Type A); 0 (Botulinum Toxins); 0 (botulinum toxin type B)

Enzyme No.: EC 3.4.21.4 (Trypsin); EC 3.4.23.1 (Pepsin A)

Record Date Created: 20020927

Record Date Completed: 20030331

?t s3/9/all

3/9/1

14054983 PMID: 9746583

Characterization of Clostridium botulinum type B neurotoxin associated with infant botulism in japan.

Kozaki S; Kamata Y; Nishiki T; Kakinuma H; Maruyama H; Takahashi H; Karasawa T; Yamakawa K; Nakamura S

Department of Veterinary Science, College of Agriculture, Osaka Prefecture University, Sakai, Osaka, Ishikawa, Japan. kozaki@center.osakafu-u.ac.jp

Infection and immunity (UNITED STATES) Oct 1998, 66 (10) p4811-6, ISSN 0019-9567 Journal Code: 0246127

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

The neurotoxin of strain 111 (111/NT) associated with type B infant botulism showed antigenic and biological properties different from that (Okra/NT) produced by a food-borne botulism-related strain, Okra. The specific toxicity of 111/NT was found to be about 10 times lower than that of Okra/NT. The monoclonal antibodies recognizing the light chain cross-reacted with both neurotoxins, whereas most of the antibodies

recognizing the **carboxyl** -terminal half of the heavy chain of Okra/NT did not react to 111/NT. Binding experiments with rat brain synaptosomes revealed that 125I-labeled 111/NT bound to a single binding site with a dissociation constant (Kd) of 2.5 nM; the value was rather lower than that (0.42 nM) of 125I-Okra/NT for the high-affinity binding site. In the lipid vesicles reconstituted with ganglioside GT1b, 125I-Okra/NT interacted with the amino-terminal domain of synaptotagmin 1 (Stg1N) or synaptotagmin 2 (Stg2N), fused with the maltose-binding protein, in the same manner as the respective full-length synaptotagmins, and the Kd values accorded with those of the low- and high-affinity binding sites in synaptosomes. However, 125I-111/NT only exhibited a low capacity for binding to the lipid vesicles containing Stg2N, but not Stg1N, in the presence of ganglioside GT1b. Moreover, synaptobrevin-2, an intracellular target protein, was digested to the same extent by the light chains of both neurotoxins in a concentration-dependent manner. These findings indicate that the 111/NT molecule possesses the receptor-recognition site structurally different from Okra/NT, probably causing a decreased specific toxicity.

Tags: Comparative Study; Human; Support, Non-U.S. Gov't

Descriptors: *Botulinum Toxins--toxicity--TO; *Botulism--microbiology--MI ; *Clostridium botulinum--pathogenicity--PY; *Metalloendopeptidases --toxicity--TO; *Neurotoxins--toxicity--TO; Animals; Botulinum Toxins --immunology--IM; Botulinum Toxins--metabolism--ME; Botulism--epidemiology --EP; Infant; Japan--epidemiology--EP; Membrane Glycoproteins--genetics--GE ; Membrane Glycoproteins--metabolism--ME; Membrane Proteins--metabolism --ME; Metalloendopeptidases--immunology--IM; Metalloendopeptidases --metabolism--ME; Nerve Tissue Proteins--genetics--GE; Nerve Tissue Proteins--metabolism--ME; Neurotoxins--immunology--IM; Neurotoxins --metabolism--ME; Protein Binding; Rats; Recombinant Proteins--genetics--GE ; Recombinant Proteins--metabolism--ME; Synaptosomes--metabolism--ME

CAS Registry No.: 0 (Botulinum Toxins); 0 (Membrane Glycoproteins); 0 (Membrane Proteins); 0 (Nerve Tissue Proteins); 0 (Neurotoxins); 0 (Recombinant Proteins); 0 (botulinum toxin type B); 0 (vesicle-associated membrane protein); 134193-27-4 (synaptotagmin)

Enzyme No.: EC 3.4.24 (Metalloendopeptidases)

Record Date Created: 19981029

Record Date Completed: 19981029

3/9/2

DIALOG(R)File 155:MEDLINE(R)

14016910 PMID: 9717738

Covalent structure of botulinum neurotoxin type B; location of sulfhydryl groups and disulfide bridge and identification of C - termini of light and heavy chains.

Antharavally B S; DasGupta B R

Department of Food Microbiology and Toxicology, University of Wisconsin, Madison 53706, USA.

Journal of protein chemistry (UNITED STATES) Jul 1998, 17 (5) p417-28, ISSN 0277-8033 Journal Code: 8217321

Contract/Grant No.: NS17742; NS; NINDS

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

Botulinum neurotoxin (NT) serotype B, produced by Clostridium botulinum (proteolytic strain), is a approximately 150-kDa single-chain polypeptide of 1291 amino acids, of which 10 are Cys residues [Whelan et al. (1992), Appl. Environ. Microbiol. 58, 2345-2354] The posttranslational modifications of the gene product were found to consist of excision of only the initiating Met residue, limited proteolysis (nicking) of the 1290-residue-long protein between Lys 440 and Ala 441, and formation of at least one disulfide bridge. The dichain (nicked) protein, in a mixture with the precursor single-chain (unnicked) molecules, was found to have a approximately 50-kDa light chain (Pro 1 through Lys 440) and a approximately 100-kDa heavy chain (Ala 441 through Glu 1290). The limited in vivo nicking of the single-chain NT to the dichain form, by protease

endogenous to the bacteria, and the nonfacile in vitro cleavage by trypsin of the Lys 440-Ala 441 bond appear to be due to the adjacent Ala 441-Pro 442 imide bond's probable cis configuration in a mixed population of molecules with cis and trans configurations. The two chains were found connected by an interchain disulfide formed by Cys 436 and Cys 445. Six other Cys residues, at positions 70, 195, 308, 777, 954, and 1277, were found in sulfhydryl form. In addition, a Cys at position 1220 or 1257 appeared to be in sulfhydryl form, hence our experimental results could not unambiguously identify presence of an intrachain disulfide bridge near the **C - terminus** of the NT. A total of 384 amino acid residues, including the 6 Cys residues at positions 70, 195, 308, 436, 445, and 1277, were identified by direct protein-chemical analysis; thus 29.7% of the protein's entire amino acid sequence predicted from the nucleotide sequence was confirmed. The 6 amino acids, residues 945-950, did not match with the sequence predicted in 1992, but did match with a later report of 1995. The above determinations were made by a combination of chemical (CNBr and acidic cleavage at Asp-Pro) and enzymatic (trypsin, clostripain, and pepsin) cleavages of the NT, and NT **carboxymethylated** with iodoacetamide (with or without 14C label), separation and isolation of the fragments by SDS-PAGE (followed by electroblotting onto PVDF membrane), and/or reversed-phase HPLC, and analyses of the fragments for the N-terminal amino acid sequences by Edman degradation and amino acid compositions.

Tags: Support, Non-U.S. Gov't; Support, U.S. Gov't, Non-P.H.S.; Support, U.S. Gov't, P.H.S.

Descriptors: *Botulinum Toxins--chemistry--CH; *Disulfides--chemistry--CH ; *Metalloendopeptidases--chemistry--CH; *Sulfhydryl Compounds--chemistry --CH; Amino Acid Sequence; Hydrolysis; Molecular Sequence Data; Protein Conformation

CAS Registry No.: 0 (Botulinum Toxins); 0 (Disulfides); 0 (Sulfhydryl Compounds); 0 (botulinum toxin type B)

Enzyme No.: EC 3.4.24 (Metalloendopeptidases)

Record Date Created: 19981029

Record Date Completed: 19981029

3/9/3

DIALOG(R)File 155:MEDLINE(R)

14015638 PMID: 9712688

Ganglioside GT1b as a complementary receptor component for Clostridium botulinum neurotoxins.

Kozaki S; Kamata Y; Watarai S; Nishiki T; Mochida S

Department of Veterinary Science, College of Agriculture, Osaka Prefecture University, Sakai, Osaka, 599-8531, USA.

Microbial pathogenesis (ENGLAND) Aug 1998, 25 (2) p91-9, ISSN 0882-4010 Journal Code: 8606191

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

Clostridium botulinum type B neurotoxin (BoNT/B) recognizes a complex of synaptotagmin II and ganglioside GT1b or GD1a as the high-affinity toxin binding site. Recombinant deletion mutants of synaptotagmin II allowed us to demonstrate that the N-terminal domain including the transmembrane region retains BoNT/B binding activity while the **C - terminal** domain is not involved in constituting the BoNT/B receptor. BoNT/B binding to reconstituted lipid vesicles containing synaptotagmin II and gangliosides showed that GT1b and GD1a confer the difference in the maximum binding capacity but not in the dissociation constant. The direct binding of GT1b to the deletion mutants revealed that the transmembrane region is required to bind GT1b, suggesting that synaptotagmin II binds to the ceramide portion of gangliosides within the plasma membrane. A monoclonal antibody against GT1b effectively inhibited not only BoNT/B binding to the reconstituted lipid vesicles and brain synaptosomes but also type A BoNT (BoNT/A) binding to brain synaptosomes. In addition, the monoclonal antibody antagonized the action of both BoNT/A and BoNT/B on synaptic transmission of rat superior cervical ganglion neurons. These results

suggest that GT1b functions as a component of the receptor complex.Copyright 1998 Academic Press

Tags: Support, Non-U.S. Gov't

Descriptors: *Botulinum Toxins--metabolism--ME; *Brain--microbiology--MI; *Clostridium botulinum--physiology--PH; *Gangliosides--physiology--PH; *Nerve Tissue Proteins--physiology--PH; *Synaptosomes--microbiology--MI; Animals; Antibodies, Monoclonal; Binding Sites; Carbohydrate Sequence; Cell Membrane--microbiology--MI; Gangliosides--analysis--AN; Gangliosides --chemistry--CH; Gangliosides--immunology--IM; Kinetics; Molecular Sequence Data; Mutagenesis; Nerve Tissue Proteins--chemistry--CH; Rats; Recombinant Fusion Proteins--metabolism--ME; Recombinant Proteins --chemistry--CH; Recombinant Proteins--metabolism--ME; Sequence Deletion

CAS Registry No.: 0 (Antibodies, Monoclonal); 0 (Botulinum Toxins); 0 (Gangliosides); 0 (Nerve Tissue Proteins); 0 (Recombinant Fusion Proteins); 0 (Recombinant Proteins); 0 (botulinum toxin type B); 141440-89-3 (synaptotagmin II); 59247-13-1 (ganglioside, GT1)

Record Date Created: 19980929

Record Date Completed: 19980929

3/9/4

13887313 PMID: 9588942

Status of Cys residues in the covalent structure of botulinum neurotoxin types A, B, and E.

Antharavally B; Tepp W; DasGupta B R

Department of Food Microbiology and Toxicology, University of Wisconsin-Madison, 53706, USA.

Journal of protein chemistry (UNITED STATES) Apr 1998, 17 (3) p187-96, ISSN 0277-8033 Journal Code: 8217321

Contract/Grant No.: NS17742; NS; NINDS

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

Clostridium botulinum neurotoxin (NT) serotypes A, B, and E have 9, 10, and 8 Cys residues, respectively, as deduced from nucleotide sequences [Whelan et al. (1992), Appl. Environ. Microbiol. 48, 2345-2354]. Each of the 150-kDa NTs has at least one disulfide; but type B, like types A and E, may have two disulfides. Using two different chemical reagents, we studied the status of the Cys residues in these three proteins after (i) the final anion exchange chromatographic step in their purification (fresh NT), (ii) 24 hr storage at 8 degrees C, (iii) precipitation with ammonium sulfate (precipitated NT), and (iv) dissolving the precipitated NT in 6 M guanidine HCl. In all three NT serotypes the number of Cys residues titrated with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) as free -SH groups varied, depending upon the absence or presence of EDTA added to the chromatography buffer, storage condition, age, and presence of the denaturant. Titration of 9.5-10 and 5.4-6.0 -SH groups in fresh NTs type B and E, respectively, indicated total and partial absence of disulfide bonds. Fewer titratable -SH groups in the precipitated NT than in the fresh NT suggested formation of disulfide and/or inaccessibility of the -SH groups due to protein's conformational change(s). When the precipitated NTs were dissolved in 6 M guanidine HCl, in the absence of any added reducing agent, all Cys residues of types B and E, and 6.4-8.3 Cys in type A NT were titratable with DTNB. Iodoacetamide modification of precipitated NT types A, B, and E **carboxymethylated** 4, 2, and 2 Cys residues, respectively; these numbers rose to 6, 9.4, and 8 when these proteins were **carboxymethylated** after dissolving in 6 M guanidine HCl in the absence of any added reducing agent. We propose that S-S- cleavage mediated by the -SH/-S-S- exchange observed in vitro after unfolding the NTs (also unfolded by 2 M guanidine HCl or urea) possibly mimicks a similar exchange process inside the endosomes, where the NTs are thought to undergo conformational changes, resulting in the reductive cleavage of the interchain disulfide between the 50-kDa light and 100-kDa heavy chain, which in turn releases the light chain and allows its egress out of the endosomes into the cytosol.

Tags: Support, Non-U.S. Gov't; Support, U.S. Gov't, Non-P.H.S.; Support, U.S. Gov't, P.H.S.

Descriptors: *Botulinum Toxin Type A--chemistry--CH; *Botulinum Toxins --chemistry--CH; *Clostridium botulinum--chemistry--CH; *Cysteine --chemistry--CH; Disulfides--chemistry--CH; Dithionitrobenzoic Acid --chemistry--CH; Protein Conformation

CAS Registry No.: 0 (Botulinum Toxin Type A); 0 (Botulinum Toxins); 0 (Disulfides); 0 (botulinum toxin type B); 0 (botulinum toxin type E); 52-90-4 (Cysteine); 69-78-3 (Dithionitrobenzoic Acid)

Record Date Created: 19980706

Record Date Completed: 19980706

3/9/5

DIALOG(R)File 155:MEDLINE(R)

13401405 PMID: 9115981

Importance of two adjacent C - terminal **sequences of SNAP-25 in exocytosis from intact and permeabilized chromaffin cells revealed by inhibition with botulinum neurotoxins A and E.**

Lawrence G W; Foran P; Mohammed N; DasGupta B R; Dolly J O

Department of Biochemistry, Imperial College of Science, Technology and Medicine, London, U.K.

Biochemistry (UNITED STATES) Mar 18 1997, 36 (11) p3061-7, ISSN 0006-2960 Journal Code: 0370623

Contract/Grant No.: NS 17742; NS; NINDS

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

Types A and E botulinum neurotoxin (BoNT) are Zn2+-requiring endoproteases which cleave nine and twenty-six residues, respectively, from the **C - terminus** of synaptosomal-associated protein of Mr = 25 kDa (SNAP-25). Involvement of SNAP-25 in the exocytosis of large dense-core vesicles in bovine adrenochromaffin cells was examined by measuring cleavage of SNAP-25 in relation to the levels of Ca2+-evoked catecholamine release from cells exposed to BoNT/A or /E, either before or after permeabilization. The dose-dependency of inhibition of exocytosis correlated closely with the extents of SNAP-25 cleavage in cells permeabilized and then treated with BoNT/E. In intact cells exposed to 66 nM BoNT/A, virtually all of the SNAP-25 was truncated, accompanied by a near-complete inhibition of exocytosis; however, after their permeabilization a significant level of secretion was recorded upon Ca2+-stimulation. Importantly, this BoNT/A-resistant release from the permeabilized cells was dramatically lowered by subsequently adding BoNT/E, which further truncated the SNAP-25 fragment (lacking the **C - terminal** nine residues) that had been produced earlier by BoNT/A. Moreover, anti-SNAP-25 IgG decreased the BoNT/A-insensitive exocytosis. When permeabilized cells were exposed to either neurotoxin, both blocked MgATP-dependent secretion but only BoNT/E attenuated the energy-independent phase. These distinct inhibitory effects of the two neurotoxins demonstrate that residues 197-205 at the **C - terminus** of SNAP-25 are absolutely essential for exocytosis from intact cells whereas even after their removal a significant proportion of the exocytotic response can be elicited from permeabilized cells, but this is reliant on amino acids 180-196. Moreover, the latter but not residues 197-205 are implicated in a late, MgATP-independent step of exocytosis, which is blocked by BoNT/E but nonsusceptible to BoNT/A.

Tags: Support, Non-U.S. Gov't; Support, U.S. Gov't, Non-P.H.S.; Support, U.S. Gov't, P.H.S.

Descriptors: *Adrenal Medulla--metabolism--ME; *Botulinum Toxin Type A --pharmacology--PD; *Botulinum Toxins--pharmacology--PD; *Chromaffin Cells --metabolism--ME; *Membrane Proteins; *Nerve Tissue Proteins--metabolism --ME; *Neurotoxins; Animals; Botulinum Toxin Type A--metabolism--ME; Botulinum Toxins--metabolism--ME; Calcium--pharmacology--PD; Catecholamines --metabolism--ME; Cattle; Cell Membrane Permeability; Chromaffin Cells --drug effects--DE; Exocytosis; Kinetics; Nerve Tissue Proteins--chemistry

--CH; Peptide Fragments--chemistry--CH; Peptide Fragments--metabolism--ME
CAS Registry No.: 0 (Botulinum Toxin Type A); 0 (Botulinum Toxins); 0 (Catecholamines); 0 (Membrane Proteins); 0 (Nerve Tissue Proteins); 0 (Neurotoxins); 0 (Peptide Fragments); 0 (botulinum toxin type B); 0 (synaptosomal-associated protein 25); 7440-70-2 (Calcium)
Record Date Created: 19970421
Record Date Completed: 19970421

3/9/6

DIALOG(R)File 155:MEDLINE(R)

13149744 PMID: 8818885

Development of novel assays for botulinum type A and B neurotoxins based on their endopeptidase activities.

Hallis B; James B A; Shone C C
Centre for Applied Microbiology and Research, Salisbury, Wiltshire, United Kingdom.

Journal of clinical microbiology (UNITED STATES) Aug 1996, 34 (8) p1934-8, ISSN 0095-1137 Journal Code: 7505564
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Subfile: INDEX MEDICUS

A novel assay method based on the endopeptidase activities of the botulinum neurotoxins has been developed and applied to the detection of botulinum type A and B toxins. An assay system developed for the detection of botulinum type B neurotoxin (BoNT/B) is based on the cleavage of a synthetic peptide substrate representing amino acid residues 60 to 94 of the intracellular target protein for the toxin, VAMP (vesicle-associated membrane protein, or synaptobrevin). In this assay system, immobilized VAMP (60-94) peptide substrate is cleaved by BoNT/B at the Gln-76-Phe-77 bond, leaving the **C - terminal** cleavage fragment on the solid phase. This fragment is then detected by the addition of an antibody-enzyme reagent which specifically recognizes the newly exposed N terminus of the cleavage product. The developed assay was specific to BoNT/B, showing no cross-reactivity with other clostridial neurotoxins, and had a sensitivity for BoNT/B of 0.6 to 4.5 ng/ml, which could be increased to 0.1 to 0.2 ng/ml by using an assay amplification system based on catalyzed reporter deposition. Trypsin treatment of BoNT/B samples, which converts the single-chain toxin to the active di-chain form, was found to increase the sensitivity of the endopeptidase assay from 5- to 10-fold. An endopeptidase assay for BoNT/A, based on the cleavage of a peptide substrate derived from the protein SNAP-25 (synaptosome-associated protein), was also developed and characterized.

Tags: Support, Non-U.S. Gov't

Descriptors: *Botulinum Toxin Type A--analysis--AN; *Botulinum Toxins --analysis--AN; *Endopeptidases--analysis--AN; *Neuromuscular Agents --analysis--AN; *Neurotoxins--analysis--AN; Horseradish Peroxidase; Immunoenzyme Techniques; Membrane Proteins--immunology--IM; Membrane Proteins--metabolism--ME; Nerve Tissue Proteins--immunology--IM; Nerve Tissue Proteins--metabolism--ME; Peptide Fragments--immunology--IM; Peptide Fragments--metabolism--ME; Substrate Specificity
CAS Registry No.: 0 (Botulinum Toxin Type A); 0 (Botulinum Toxins); 0 (Membrane Proteins); 0 (Nerve Tissue Proteins); 0 (Neuromuscular Agents); 0 (Neurotoxins); 0 (Peptide Fragments); 0 (botulinum toxin type B); 0 (synaptosomal-associated protein 25); 0 (vesicle-associated membrane protein)
Enzyme No.: EC 1.11.1.- (Horseradish Peroxidase); EC 3.4.- (Endopeptidases)
Record Date Created: 19961210
Record Date Completed: 19961210

3/9/7

DIALOG(R)File 155:MEDLINE(R)

12515819 PMID: 14561084

Development of potent inhibitors of botulinum neurotoxin type B.

Anne Christine; Turcaud Serge; Quancard Jean; Teffo Franck; Meudal Herve; Fournie-Zaluski Marie-Claude; Roques Bernard P

Departement de Pharmacochimie Moleculaire et Structurale, INSERM U266--CNRS FRE2463, UFR des Sciences Pharmaceutiques et Biologiques 4, Avenue de l'Observatoire--75270 Paris Cedex 06, France.

Journal of medicinal chemistry (United States) Oct 23 2003, 46 (22) p4648-56, ISSN 0022-2623 Journal Code: 9716531

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

Botulinum neurotoxins are the most potent toxins known to date. They are zinc-metalloproteases able to cleave selectively an essential component of neurotransmitter exocytosis, causing the syndrome of botulism characterized by a flaccid paralysis. There is a great interest in designing antagonists of the action of these toxins. One way is to inhibit their catalytic activity. In this study, we report the design of such inhibitors directed toward BoNT/B. A study of the S(1) subsite specificity, using several beta-amino thiols, has shown that this subsite prefers a p- **carboxybenzyl** moiety. The specificity of the S(1)' and S(2)' subsites was studied using two libraries of pseudotripeptides containing the S(1) synthon derived from the best beta-amino thiol tested. Finally, a selection of various non natural amino acids for the recognition of the "prime" domain led to the most potent inhibitor of BoNT/B described to date with a K(i) value of 20 nM.

Descriptors: *Benzoic Acids--chemical synthesis--CS; *Botulinum Toxins --antagonists and inhibitors--AI; *Metalloendopeptidases--antagonists and inhibitors--AI; *Oligopeptides--chemical synthesis--CS; *Protease Inhibitors--chemical synthesis--CS; Benzoic Acids--chemistry--CH; Botulinum Toxins--chemistry--CH; Metalloendopeptidases--chemistry--CH; Models, Molecular; Oligopeptides--chemistry--CH; Protease Inhibitors--chemistry--CH ; Structure-Activity Relationship

CAS Registry No.: 0 (4-(2-amino-3-((1-(2-benzo(b)thiophen-3-yl)-1-(benzylcarbamoyl)ethylcarbamoyl-2-(biphenyl-4-yl)ethylcarbamoyl)-3-sulfanylpropyl))benzoic acid); 0 (Benzoic Acids); 0 (Botulinum Toxins); 0 (Oligopeptides); 0 (Protease Inhibitors); 0 (botulinum toxin type B)

Enzyme No.: EC 3.4.24 (Metalloendopeptidases)

Record Date Created: 20031016

Record Date Completed: 20031112

3/9/8

12190421 PMID: 12527421

Sequence of the gene for Clostridium botulinum type B neurotoxin associated with infant botulism, expression of the C - terminal **half of heavy chain and its binding activity.**

Ihara Hideshi; Kohda Tomoko; Morimoto Fumihiro; Tsukamoto Kentaro; Karasawa Tadahiro; Nakamura Shinichi; Mukamoto Masafumi; Kozaki Shunji

Department of Earth and Life Sciences, College of Integrated Arts and Sciences, Osaka Prefecture University, Sakai, Japan.

Biochimica et biophysica acta (Netherlands) Jan 3 2003, 1625 (1) p19-26, ISSN 0006-3002 Journal Code: 0217513

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

Previously, we demonstrated that the neurotoxin of strain 111 (111/NT) associated with type B infant botulism showed antigenic and biological properties different from that (Okra/NT) produced by a foodborne botulism-related strain, Okra. In this study, the neurotoxin genes of 111/NT and Okra/NT were amplified and the sequences determined. The

nucleotide sequences of the genes for both neurotoxins possessed an open reading frame of 3873 bp that encoded 1291 amino acids. The identities of nucleotide sequences and amino acid sequences were 97.6% and 95.7%, respectively. The ratio of nonsynonymous to synonymous substitutions was 0.47. The amino acid substitutions between 111/NT and Okra/NT occurred mainly in the domain of the **C - terminal** half of heavy chain (H(C)) responsible for binding to its receptor complex of protein and ganglioside. To characterize the binding capability of the H(C), recombinant genes for the H(C) and two hybrid H(C) in which one half of Okra/NT was replaced by the homologous half of 111/NT were constructed and expressed in Escherichia coli. The binding activity of the recombinant H(C) of 111/NT to the protein receptor synaptotagmin II, in the presence of ganglioside GT1b, was 4.2-fold less than Okra/NT, consistent with the corresponding two NTs. The use of hybrid H(C) revealed that mutation of 23 residues in **carboxy** terminal half of H(C) (1029-1291) of Okra/NT could be attributed to the lower binding activity of 111/NT and thus the differences in binding affinity between the two BoNT/B.

Tags: Human; Support, Non-U.S. Gov't

Descriptors: *Botulinum Toxins--genetics--GE; *Clostridium botulinum --genetics--GE; Amino Acid Sequence; Base Sequence; Botulinum Toxins --biosynthesis--BI; Botulinum Toxins--metabolism--ME; Botulism--metabolism --ME; Child, Preschool; Clostridium botulinum--metabolism--ME; Infant; Molecular Sequence Data; Protein Binding--physiology--PH; Recombinant Proteins--genetics--GE; Recombinant Proteins--metabolism--ME; Sequence Alignment

CAS Registry No.: 0 (Botulinum Toxins); 0 (Recombinant Proteins); 0 (botulinum toxin type B)

Record Date Created: 20030115

Record Date Completed: 20030305

3/9/9

DIALOG(R)File 155:MEDLINE(R)

12178292 PMID: 12514008

Fluorigenic substrates for the protease activities of botulinum neurotoxins, serotypes A, B, and F.

Schmidt James J; Stafford Robert G

Toxinology and Aerobiology Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland 21702-5011, USA. james.scmidt@det.amedd.army.mil

Applied and environmental microbiology (United States) Jan 2003, 69 (1) p297-303, ISSN 0099-2240 Journal Code: 7605801

Erratum in Appl Environ Microbiol. 2003 May;69(5) 3025

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

The seven botulinum neurotoxins (BoNTs) are zinc metalloproteases that cleave neuronal proteins involved in neurotransmitter release and are among the most toxic natural products known. High-throughput BoNT assays are needed for use in antibotulinum drug discovery and to characterize BoNT protease activities. Compared to other proteases, BoNTs exhibit unusually stringent substrate requirements with respect to amino acid sequences and polypeptide lengths. Nonetheless, we have devised a strategy for development of fluorigenic BoNT protease assays, based on earlier structure-function studies, that has proven successful for three of the seven serotypes: A, B, and F. In synthetic peptide substrates, the P(1) and P(3)' residues were substituted with 2,4-dinitrophenyl-lysine and S-(N-[4-methyl-7-dimethylamino-coumarin-3-yl]- **carboxamidomethyl**)-cysteine , respectively. By monitoring the BoNT-catalyzed increase in fluorescence over time, initial hydrolysis rates could be obtained in 1 to 2 min when BoNT concentrations were 60 ng/ml (about 1 nM) or higher. Each BoNT cleaved its fluorigenic substrate at the same location as in the neuronal target protein, and kinetic constants indicated that the substrates were selective and efficient. The fluorigenic assay for BoNT B was used to characterize a new competitive inhibitor of BoNT B protease activity with a K(i) value of

4 micro M. In addition to real-time activity measurements, toxin concentration determinations, and kinetic studies, the BoNT substrates described herein may be directly incorporated into automated high-throughput assay systems to screen large numbers of compounds for potential antibotulinum drugs.
Descriptors: *Botulinum Toxins--metabolism--ME; *Endopeptidases --metabolism--ME; *Fluorescent Dyes--chemical synthesis--CS; *Fluorescent Dyes--metabolism--ME; *Peptides--chemical synthesis--CS; *Peptides --metabolism--ME; Amino Acid Sequence; Botulinum Toxin Type A--metabolism --ME; Drug Evaluation, Preclinical; Fluorescent Dyes--chemistry--CH; Molecular Sequence Data; Peptides--chemistry--CH; Protease Inhibitors --metabolism--ME; Protease Inhibitors--pharmacology--PD; Substrate Specificity
CAS Registry No.: 0 (Botulinum Toxin Type A); 0 (Botulinum Toxins); 0 (Fluorescent Dyes); 0 (Peptides); 0 (Protease Inhibitors); 0 (botulinum toxin type B); 0 (botulinum toxin type F)
Enzyme No.: EC 3.4.- (Endopeptidases)
Record Date Created: 20030106
Record Date Completed: 20030321

3/9/10
DIALOG(R)File 155:MEDLINE(R)

12025856 PMID: 12270602
Recovery of intracellular recombinant proteins from the yeast Pichia pastoris by cell permeabilization.
Shepard Scot R; Stone Charmaine; Cook Susan; Bouvier Anne; Boyd Gregory; Weatherly Gresham; Lydiard Debra; Schrimsher Jeffrey
Diosynth RTP, Inc, 3000 Weston Parkway, Cary, NC 27513, USA. scot.shepard@diosynth-rtp.com
Journal of biotechnology (Netherlands) Oct 23 2002, 99 (2) p149-60, ISSN 0168-1656 Journal Code: 8411927
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Subfile: INDEX MEDICUS
A cell permeabilization method for the release of intracellular proteins from microbial cells was developed. The method was applied to the recovery of recombinant botulinum neurotoxin fragments, expressed intracellularly in the yeast Pichia pastoris, by suspending the cells in an aqueous solution containing N,N-dimethyltetradecylamine. For the botulinum neurotoxin serotype B **C - terminal** heavy chain fragment, 1.8 mg g(-1) biomass were recovered. For the botulinum neurotoxin serotype A **C - terminal** heavy chain fragment, 3.7 mg g(-1) biomass were recovered. The concentration of recombinant protein in the cell extracts remained stable for up to 48 and 24 h for the serotype B and serotype A fragments, respectively. The permeabilization method was compared with high-pressure homogenization; the permeabilization method proved to be both more selective and more efficient. Copyright 2002 Elsevier Science B.V.
Tags: Comparative Study; Support, Non-U.S. Gov't; Support, U.S. Gov't, Non-P.H.S.
Descriptors: *Botulinum Toxin Type A--isolation and purification--IP; *Botulinum Toxins--isolation and purification--IP; *Cell Membrane Permeability--drug effects--DE; *Pichia--metabolism--ME; *Recombinant Proteins--isolation and purification--IP; Botulinum Toxin Type A --biosynthesis--BI; Botulinum Toxin Type A--genetics--GE; Botulinum Toxins --biosynthesis--BI; Botulinum Toxins--genetics--GE; Enzyme Stability; Pichia--genetics--GE; Pichia--ultrastructure--UL; Pressure; Recombinant Proteins--biosynthesis--BI; Sensitivity and Specificity
CAS Registry No.: 0 (Botulinum Toxin Type A); 0 (Botulinum Toxins); 0 (Recombinant Proteins); 0 (botulinum toxin type B)
Record Date Created: 20020924
Record Date Completed: 20021115

3/9/11

DIALOG(R)File 155:MEDLINE(R)

11882640 PMID: 12077369

Ca2+-induced changes in SNAREs and synaptotagmin I correlate with triggered exocytosis from chromaffin cells: insights gleaned into the signal transduction using trypsin and botulinum toxins.

Lawrence Gary W; Dolly J Oliver

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Journal of cell science (England) Jul 1 2002, 115 (Pt 13) p2791-800, ISSN 0021-9533 Journal Code: 0052457

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

Ca2+-triggered catecholamine exocytosis from chromaffin cells involves SNAP-25, synaptobrevin and syntaxin (known as SNAREs). Synaptotagmin I has been implicated as the Ca2+-sensor because it binds Ca2+, and this enhances its binding to syntaxin, SNAP-25 and phospholipids in vitro. However, most of these interactions are only mediated by [Ca2+]i two orders of magnitude higher than that needed to elicit secretion. Thus, the Ca2+ sensitivities of synaptotagmin I and the other SNAREs were quantified in situ. Secretion elicited from permeabilised cells by microM Ca2+ was accompanied, with almost identical Ca2+ dependencies, by changes in synaptotagmin I, SNAP-25, syntaxin and synaptobrevin that rendered them less susceptible to trypsin. The majority of the trypsin-resistant SNAREs were not associated with SDS-resistant complexes. None of these proteins acquired trypsin resistance in cells rendered incompetent for exocytosis by run-down. Removal of nine **C - terminal** residues from SNAP-25 by botulinum toxin A reduced both exocytosis and the SNAREs' acquisition of trypsin resistance but did not alter the Ca2+ sensitivity, except for synaptotagmin I. Even after synaptobrevin had been cleaved by botulinum toxin B, all the other proteins still responded to Ca2+. These data support a model whereby Ca2+ is sensed, probably by synaptotagmin I, and the signal passed to syntaxin and SNAP-25 before they interact with synaptobrevin.

Descriptors: *Calcium--metabolism--ME; *Calcium Signaling--physiology--PH; *Cell Membrane--metabolism--ME; *Chromaffin Cells--secretion--SE; *Exocytosis--physiology--PH; *Membrane Glycoproteins--metabolism--ME; *Membrane Proteins--metabolism--ME; *Nerve Tissue Proteins--metabolism--ME; Adenosine Triphosphate--diagnostic use--DU; Adrenal Medulla; Animals; Botulinum Toxins--pharmacology--PD; Calcium--pharmacology--PD; Calcium Signaling--drug effects--DE; Catecholamines--metabolism--ME; Catecholamines--secretion--SE; Cattle; Cell Membrane--drug effects--DE; Cells, Cultured; Chromaffin Cells--drug effects--DE; Chromaffin Cells--metabolism--ME; Dose-Response Relationship, Drug; Endopeptidase K--diagnostic use--DU; Exocytosis--drug effects--DE; Membrane Glycoproteins--drug effects--DE; Membrane Proteins--drug effects--DE; Nerve Tissue Proteins--drug effects--DE; Secretory Vesicles--drug effects--DE; Secretory Vesicles--metabolism--ME; Signal Transduction--drug effects--DE; Signal Transduction--physiology--PH; Trypsin--diagnostic use--DU

CAS Registry No.: 0 (Botulinum Toxins); 0 (Catecholamines); 0 (Membrane Glycoproteins); 0 (Membrane Proteins); 0 (Nerve Tissue Proteins); 0 (SNAP receptor); 0 (botulinum toxin type B); 0 (synaptosomal-associated protein 25); 0 (vesicle-associated membrane protein); 134193-27-4 (synaptotagmin); 157546-56-0 (syntaxin); 56-65-5 (Adenosine Triphosphate); 7440-70-2 (Calcium)

Enzyme No.: EC 3.4.21.4 (Trypsin); EC 3.4.21.64 (Endopeptidase K)

Record Date Created: 20020621

Record Date Completed: 20021126

3/9/12

DIALOG(R)File 155:MEDLINE(R)

11386947 PMID: 11478959

Botulinum neurotoxin types B and E: purification, limited proteolysis by endoproteinase Glu-C and pepsin, and comparison of their identified cleaved sites relative to the three-dimensional structure of type A neurotoxin.

Prabakaran S; Tepp W; DasGupta B R

Department of Food Microbiology and Toxicology, University of Wisconsin, Madison, 53706, USA.

Toxicon - official journal of the International Society on Toxinology (England) Oct 2001, 39 (10) p1515-31, ISSN 0041-0101 Journal Code: 1307333

Contract/Grant No.: NS 17742; NS; NINDS

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

Botulinum neurotoxin (NT) serotypes B and E are approximately 150 kDa proteins. Isolated from the liquid culture of Clostridium botulinum the NT type E is a single chain protein while the NT type B, from the proteolytic strain of the bacteria, is a mixture of dichain (nicked within a disulfide loop located about one-third the way from the N-terminus to the **C - terminus**) protein and its precursor single-chain protein. Endoproteinase Glu-C (EC 3.4.21.19) and pepsin (EC 3.4.23.1) were used for controlled digestion of NT types B and E; the amino acid residues flanking many of the cleavable peptide bonds were identified and the corresponding proteolytic fragments partially characterized. Chemical identification of 82 and 108 residues of types B and E NT, respectively, revealed that the residue 738 and 1098 in type E NT, identified as Leu and Asn, respectively, differ from those deduced from nucleotide sequences. Several fragments overlapped spanning various segments of the NT's functional domains; they appear to have potential for structure-function studies of the NT. The cleavage sites were compared with the previously determined proteolyzed sites on NT types A and E. The cleavage sites of the NT types A, B and E, all exposed on the protein surface, were scrutinized in the context of the three-dimensional structure of crystallized NT type A [Lacy, D.B., Stevens, R.C., 1999. J. Mol. Biol. 291, 1091-1104]. Detailed procedures for isolation of pure NT types B and E in large quantities (average yield 92 and 62 mg, respectively) suitable for crystallization are reported.

Tags: Comparative Study; Support, Non-U.S. Gov't; Support, U.S. Gov't, P.H.S.

Descriptors: *Binding Sites; *Botulinum Toxin Type A--metabolism--ME; *Botulinum Toxins--isolation and purification--IP; *Botulinum Toxins --metabolism--ME; *Clostridium botulinum--pathogenicity--PY; *Pepsin A --metabolism--ME; *Peptide Fragments--metabolism--ME; *Serine Endopeptidases--metabolism--ME; Amino Acid Sequence; Botulinum Toxin Type A --chemistry--CH; Chromatography, Agarose; Chromatography, DEAE-Cellulose; Crystallography, X-Ray; Electrophoresis, Polyacrylamide Gel; Neuromuscular Agents--metabolism--ME; Peptide Fragments--chemistry--CH; Peptide Fragments--isolation and purification--IP; Protein Conformation; Sequence Alignment

CAS Registry No.: 0 (Botulinum Toxin Type A); 0 (Botulinum Toxins); 0 (Neuromuscular Agents); 0 (Peptide Fragments); 0 (botulinum toxin type B); 0 (botulinum toxin type E)

Enzyme No.: EC 3.4.21 (Serine Endopeptidases); EC 3.4.21.19 (glutamyl endopeptidase); EC 3.4.23.1 (Pepsin A)

Record Date Created: 20010731

Record Date Completed: 20011004

3/9/13

10496744 PMID: 10594895

Peptides that mimic the carboxy -terminal domain of SNAP-25 block acetylcholine release at an Aplysia synapse.

Apland J P; Biser J A; Adler M; Ferrer-Montiel A V; Montal M; Canaves J M ; Filbert M G

Neurotoxicology Branch, US Army Medical Research Institute of Chemical Defense, 3100 Ricketts Point Road, Aberdeen Proving Ground, MD 21010-5400,

USA.
Journal of applied toxicology - JAT (ENGLAND) Dec 1999, 19 Suppl 1 pS23-6, ISSN 0260-437X Journal Code: 8109495
Erratum in J Appl Toxicol 2000 Nov-Dec;20(6) 499
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Subfile: INDEX MEDICUS

Botulinum neurotoxin serotypes A and E (BoNT/A and BoNT/E) block neurotransmitter release, presumably by cleaving SNAP-25, a protein involved in docking of synaptic vesicles with the presynaptic plasma membrane. Three excitation-secretion uncoupling peptides (ESUPs), which mimic the **carboxy** -terminal domain of SNAP-25 and span or adjoin the cleavage sites for BoNT/A and BoNT/E, also inhibit transmitter release from permeabilized bovine chromaffin cells. In this study, these peptides were tested for effects on acetylcholine (ACh) release at an identified cholinergic synapse in isolated buccal ganglia of Aplysia californica. The presynaptic neuron was stimulated electrically to elicit action potentials. The postsynaptic neuron was voltage-clamped, and evoked inhibitory postsynaptic currents (IPSCs) were recorded. The ESUPs were pressure-injected into the presynaptic neuron, and their effects on the amplitude of the IPSCs were studied. Acetylcholine release from presynaptic cells, as measured by IPSC amplitudes, was gradually inhibited by the ESUPs. All three peptides caused ca. 40% reduction in IPSC amplitude in 2 h. Random-sequence peptides of the same amino acid composition had no effect. Injection of BoNT/E, in contrast, caused ca. 50% reduction in IPSC amplitude in 30 min and almost complete inhibition in 2 h. These results are the first demonstration that ESUPs block neuronal cholinergic synaptic transmission. They are consistent with the concept that ESUPs compete with the intact SNAP-25 for binding with other fusion proteins, thus inhibiting stimulus-evoked exocytosis of neurotransmitter.

Descriptors: *Acetylcholine--secretion--SE; *Botulinum Toxins--toxicity --TO; *Membrane Proteins; *Nerve Tissue Proteins--pharmacology--PD; *Peptide Fragments--pharmacology--PD; *Synapses--drug effects--DE; Action Potentials--drug effects--DE; Animals; Aplysia; Synapses--secretion--SE

CAS Registry No.: 0 (Botulinum Toxins); 0 (Membrane Proteins); 0 (Nerve Tissue Proteins); 0 (Peptide Fragments); 0 (botulinum toxin type B); 0 (synaptosomal-associated protein 25); 51-84-3 (Acetylcholine)

Record Date Created: 20000321
Record Date Completed: 20000321

3/9/14
DIALOG(R)File 155:MEDLINE(R)

10496741 PMID: 10594892

Evaluation of phosphoramidon and three synthetic phosphonates for inhibition of botulinum neurotoxin B catalytic activity.

Adler M; Nicholson J D; Starks D F; Kane C T; Cornille F; Hackley B E
Neurotoxicology Branch, Pharmacology Division, US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground MD, 21010, USA.

Journal of applied toxicology - JAT (ENGLAND) Dec 1999, 19 Suppl 1 pS5-S11, ISSN 0260-437X Journal Code: 8109495
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Subfile: INDEX MEDICUS

Three putative metalloprotease inhibitors were synthesized and tested for their ability to inhibit the catalytic activity of botulinum neurotoxin B light chain (BoNT/B LC). The compounds were designed to emulate the naturally occurring metalloprotease inhibitor phosphoramidon, which has been reported to be a weak antagonist of BoNT/B action. All three analogs contained the dipeptide Phe-Glu in place of Leu-Trp of phosphoramidon and possessed a phenyl, ethyl or methyl group in place of the rhamnose sugar of the parent compound. The inhibitors were evaluated in a cell-free assay based on the detection of a fluorescent product following cleavage of a

50-mer synaptobrevin peptide ([Pya(88)] S 39-88) by BoNT/B LC. This peptide corresponds to the hydrophilic core of synaptobrevin-2 and contains a fluorescent analog L-pyrenylalanine (Pya) in place of Tyr(88). Cleavage of [Pya(88)] S 39-88 by BoNT/B LC gives rise to fragments of 38 and 12 amino acid residues. Quantification of BoNT/B-mediated substrate cleavage was achieved by separating the 12-mer fragment (FETSAAKLKRK-Pya) that contains the **C - terminal** fluorophore and measuring fluorescence at 377 nm. The results indicate that the phenyl-substituted synthetic compound ICD 2821 was slightly more active than phosphoramidon, but analogs with methyl or ethyl substitutions were relatively inactive. These findings suggest that phosphonate monoesters may be useful for providing insights into the structural requirement of BoNT/B protease inhibitors.

Tags: Support, U.S. Gov't, Non-P.H.S.

Descriptors: *Botulinum Toxins--antagonists and inhibitors--AI; *Glycopeptides--pharmacology--PD; *Metalloendopeptidases --antagonists and inhibitors--AI; *Phosphonic Acids--pharmacology--PD; *Protease Inhibitors --pharmacology--PD; Catalysis; Structure-Activity Relationship; Zinc Sulfate--pharmacology--PD

CAS Registry No.: 0 (Botulinum Toxins); 0 (Glycopeptides); 0 (Phosphonic Acids); 0 (Protease Inhibitors); 0 (botulinum toxin type B); 36357-77-4 (phosphoramidon); 7733-02-0 (Zinc Sulfate)

Enzyme No.: EC 3.4.24 (Metalloendopeptidases)

Record Date Created: 20000321

Record Date Completed: 20000321

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15mar04 16:46:20 User228206 Session D2136.2

$5.47 1.709 DialUnits File155

$3.57 17 Type(s) in Format 9

$3.57 17 Types

$9.04 Estimated cost File155

$0.50 TELNET

$9.54 Estimated cost this search

$9.54 Estimated total session cost 1.861 DialUnits

Status: Signed Off. (2 minutes)

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econnected in file MEDICINE 15mar04 16:58:25
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* ALL NEW CURRENT YEAR RANGES HAVE BEEN * * *
* * * INSTALLED   * * *

SYSTEM:OS  - DIALOG OneSearch
  File   5:Biosis Previews(R)  1969-2004/Mar W1
         (c) 2004 BIOSIS
  File  34:SciSearch(R) Cited Ref Sci  1990-2004/Mar W1
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  File  35:Dissertation Abs Online  1861-2004/Feb
         (c) 2004 ProQuest Info&Learning
  File  48:SPORTDiscus  1962-2004/Mar
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         2001 (c) Action Potential
  File  94:JICST-EPlus  1985-2004/Mar W1
         (c)2004 Japan Science and Tech Corp(JST)
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  File 149:TGG Health&Wellness DB(SM)  1976-2004/Mar W1
         (c) 2004 The Gale Group
  File 155:MEDLINE(R)  1966-2004/Mar W1
         (c) format only 2004 The Dialog Corp.
*File 155: Medline has been reloaded. Accession numbers
have changed. Please see HELP NEWS 154 for details.
  File 156:ToxFile  1965-2004/Mar W2
         (c) format only 2004 The Dialog Corporation
  File 159:Cancerlit  1975-2002/Oct
         (c) format only 2002 Dialog Corporation
*File 159: Cancerlit ceases updating with immediate effect.
Please see HELP NEWS.
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         (c) 2004 CAB International
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         (c) 2004 Reed Business Information Ltd.
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information.
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         (c) 2004 American Chemical Society
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Alert feature enhanced for multiple files, etc. See HELP ALERT.
  File 434:SciSearch(R) Cited Ref Sci  1974-1989/Dec
         (c) 1998 Inst for Sci Info
  File 444:New England Journal of Med.  1985-2004/Mar W2
         (c) 2004 Mass. Med. Soc.
  File 467:ExtraMED(tm)  2000/Dec
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*File 467: For information about updating status please see Help News467.

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Cost is in DialUnits
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Set	Items	Description
S1	148720	HEAVY? (3N) CHAIN?
S2	1433034	CTERMINAL? OR CARBOX? OR (C (2N) TERMIN?)
S3	49127	BOTULIN?
S4	5098406	EPITOP? OR ANTIGEN? OR MONOCLONAL? OR MAP?
S5	63	S1 AND S2 AND S3 AND S4
S6	22	RD (unique items)

?t s6/9/all

6/9/1 (Item 1 from file: 5)

0014138262 BIOSIS NO.: 200300096981

Sequence of the gene for Clostridium botulinum **type B neurotoxin associated with infant botulism, expression of the** C - terminal **half of** heavy chain **and its binding activity.**

AUTHOR: Ihara Hideshi; Kohda Tomoko; Morimoto Fumihiro; Tsukamoto Kentaro; Karasawa Tadahiro; Nakamura Shinichi; Mukamoto Masafumi; Kozaki Shunji (Reprint)
AUTHOR ADDRESS: Department of Veterinary Science, Graduate School of Agriculture and Biological Sciences, Osaka Prefecture University, 1-1 Gakuen-cho, Sakai, Osaka, 599-8531, Japan**Japan
AUTHOR E-MAIL ADDRESS: kozaki@center.osakafu-u.ac.jp

JOURNAL: Biochimica et Biophysica Acta 1625 (1): p19-26 3 January, 2003 2003
MEDIUM: print
ISSN: 0006-3002 _(ISSN print)
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: Previously, we demonstrated that the neurotoxin of strain 111 (111/NT) associated with type B infant botulism showed **antigenic** and biological properties different from that (Okra/NT) produced by a foodborne botulism-related strain, Okra. In this study, the neurotoxin genes of 111/NT and Okra/NT were amplified and the sequences determined. The nucleotide sequences of the genes for both neurotoxins possessed an open reading frame of 3873 bp that encoded 1291 amino acids. The identities of nucleotide sequences and amino acid sequences were 97.6% and 95.7%, respectively. The ratio of nonsynonymous to synonymous substitutions was 0.47. The amino acid substitutions between 111/NT and Okra/NT occurred mainly in the domain of the **C - terminal** half of **heavy chain** (HC) responsible for binding to its receptor complex of protein and ganglioside. To characterize the binding capability of the HC, recombinant genes for the HC and two hybrid HC in which one half of Okra/NT was replaced by the homologous half of 111/NT were constructed and expressed in Escherichia coli. The binding activity of the recombinant HC of 111/NT to the protein receptor synaptotagmin II, in the presence of ganglioside GT1b, was 4.2-fold less than Okra/NT, consistent with the corresponding two NTs. The use of hybrid HC revealed that mutation of 23 residues in **carboxy** terminal half of HC (1029-1291) of Okra/NT could be attributed to the lower binding activity of 111/NT and thus the differences in binding affinity between the two BoNT/B.

DESCRIPTORS:
MAJOR CONCEPTS: Infection; Molecular Genetics--Biochemistry and Molecular Biophysics; Nervous System--Neural Coordination; Toxicology
BIOSYSTEMATIC NAMES: Enterobacteriaceae--Facultatively Anaerobic Gram-Negative Rods, Eubacteria, Bacteria, Microorganisms; Endospore-forming Gram-Positives--Eubacteria, Bacteria, Microorganisms; Hominidae--Primates, Mammalia, Vertebrata, Chordata, Animalia

ORGANISMS: Escherichia coli (Enterobacteriaceae); Clostridium **botulinum** (Endospore-forming Gram-Positives)--pathogen, strain-111, strain-Okra; human (Hominidae)--infant, patient
COMMON TAXONOMIC TERMS: Bacteria; Eubacteria; Microorganisms; Animals; Chordates; Humans; Mammals; Primates; Vertebrates
DISEASES: infant botulism--bacterial disease, toxicity
CHEMICALS & BIOCHEMICALS: neurotoxin; protein; ganglioside; synaptotagmin II; **heavy chain -- C - terminal**
GENE NAME: Clostridium **botulinum** neurotoxin gene (Endospore-forming Gram-Positives)
CONCEPT CODES:
03502 Genetics - General
03508 Genetics - Human
10064 Biochemistry studies - Proteins, peptides and amino acids
20504 Nervous system - Physiology and biochemistry
20506 Nervous system - Pathology
22501 Toxicology - General and methods
25000 Pediatrics
31000 Physiology and biochemistry of bacteria
31500 Genetics of bacteria and viruses
36002 Medical and clinical microbiology - Bacteriology
BIOSYSTEMATIC CODES:
06702 Enterobacteriaceae
07810 Endospore-forming Gram-Positives
86215 Hominidae

6/9/2 (Item 2 from file: 5)

0011687277 BIOSIS NO.: 199800481524
Characterization of Clostridium botulinum **type B neurotoxin associated with infant botulism in Japan**
AUTHOR: Kozaki Shunji (Reprint); Kamata Yoichi; Nishiki Tei-Ichi; Kakinuma Hiroaki; Maruyama Hiromi; Takahashi Hiroaki; Karasawa Tadahiro; Yamakawa Kiyotaka; Nakamura Shinichi
AUTHOR ADDRESS: Dep. Veterinary Sci., Coll. Agric., Osaka Prefecture Univ., 1-1 Gakuen-cho, Sakai, Osaka 599-8531, Japan**Japan
JOURNAL: Infection and Immunity 66 (10): p4811-4816 Oct., 1998 1998
MEDIUM: print
ISSN: 0019-9567
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: The neurotoxin of strain 111 (111/NT) associated with type B infant botulism showed **antigenic** and biological properties different from that (Okra/NT) produced by a food-borne botulism-related strain, Okra. The specific toxicity of 111/NT was found to be about 10 times lower than that of Okra/NT. The **monoclonal** antibodies recognizing the light chain cross-reacted with both neurotoxins, whereas most of the antibodies recognizing the **carboxyl** -terminal half of the **heavy chain** of Okra/NT did not react to 111/NT. Binding experiments with rat brain synaptosomes revealed that 125I-labeled 111/NT bound to a single binding site with a dissociation constant (Kd) of 2.5 nM; the value was rather lower than that (0.42 nM) of 125I-Okra/NT for the high-affinity binding site. In the lipid vesicles reconstituted with ganglioside GT1b, 125I-Okra/NT interacted with the amino-terminal domain of synaptotagmin 1 (Stg1N) or synaptotagmin 2 (Stg2N), fused with the maltose-binding protein, in the same manner as the respective full-length synaptotagmins, and the Kd values accorded with those of the low- and high-affinity binding sites in synaptosomes. However, 125I-111/NT only exhibited a low capacity for binding to the lipid vesicles containing Stg2N, but not Stg1N, in the presence of ganglioside GT1b. Moreover, synaptobrevin-2, an intracellular target protein, was digested to the same extent by the light chains of both neurotoxins in a concentration-dependent manner. These findings indicate that the 111/NT molecule possesses the receptor-recognition site structurally different from Okra/NT, probably

causing a decreased specific toxicity.

DESCRIPTORS:
MAJOR CONCEPTS: Epidemiology--Population Studies; Infection
BIOSYSTEMATIC NAMES: Endospore-forming Gram-Positives--Eubacteria, Bacteria, Microorganisms
ORGANISMS: Clostridium- **botulinum** (Endospore-forming Gram-Positives)--strain-111
COMMON TAXONOMIC TERMS: Bacteria; Eubacteria; Microorganisms
DISEASES: infant botulism--bacterial disease, toxicity
CHEMICALS & BIOCHEMICALS: Clostridium- **botulinum** type B neurotoxin--characterization
GEOGRAPHICAL NAME: Japan (Palearctic region)
CONCEPT CODES:
36001 Medical and clinical microbiology - General and methods
04500 Mathematical biology and statistical methods
20501 Nervous system - General and methods
25000 Pediatrics
37056 Public health: epidemiology - Miscellaneous
05500 Social biology and human ecology
BIOSYSTEMATIC CODES:
07810 Endospore-forming Gram-Positives

6/9/3 (Item 3 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

0011164960 BIOSIS NO.: 199799799020
Botulinum **neurotoxin type A: Limited proteolysis by endoproteinase Glu-C and alpha-chymotrypsin enhanced following reduction; Identification of the cleaved sites and fragments**
AUTHOR: Beecher Douglas J; Dasgupta Bibhuti R (Reprint)
AUTHOR ADDRESS: Dep. Food Microbiol. Toxicol., Univ. Wisconsin, Madison, WI 53706, USA**USA
JOURNAL: Journal of Protein Chemistry 16 (7): p701-712 1997 1997
ISSN: 0277-8033
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: **Botulinum** neurotoxin (NT) serotype A is a apprx 150-kDa dichain protein. Posttranslational nicking of the single-chain NT (residues Pro 1-Leu 1295) by the protease(s) endogenous to Clostridium **botulinum** excises 10 residues, leaving Pro 1-Lys 437 and Ala 448-Leu 1295 in the apprx 50-kDa light (L) and apprx 100-kDa **heavy** (H) **chains**, respectively, connected by a Cys 429-Cys 453 disulfide and noncovalent bonds (Krieglstein et al. (1994), J. Protein Chem. 13, 49-57). The L chain is a metalloprotease, while the amino- and **carboxy**-terminal halves of the H chain have channel-forming and receptor-binding activities, respectively (Montecucco and Schiavo (1995), Q. Rev. Biophys. 28, 423-472). Endoproteinase Glu-C and alpha-chymotrypsin were used for controlled digestion at pH 7.4 of the apprx 150-kDa dichain NT and the isolated apprx 100-kDa H chain (i.e., freed from the L chain) in order to **map** the cleavage sites and isolate the proteolytic fragments. The dichain NT appeared more resistant to cleavage by endoproteinase Glu-C than the isolated H chain. In contrast, the NT with its disulfide(s) reduced showed rapid digestion of both chains, including a cleavage between Glu 251 and Met 252 (resulting in apprx 30- and apprx 20-kDa fragments of the L chain) which was not noted unless the NT was reduced. Interestingly, an adjacent bond, Tyr 249-Tyr 250, was noted earlier (DasGupta and Foley (1989), Biochimie 71, 1193-1200) to undergo "self-cleavage" following reductive separation of the L chain from the H chain. The site Tyr-Tyr-Glu-Met (residues 249-252) appears to become exposed following reduction of Cys 429-Cys 453 disulfide. Identification of Glu 669-Ile 670 and Tyr 683-Ile 684 as protease-susceptible sites demonstrated for the first time that at least two peptide bonds in the segment of the H chain (residues 659-684), part of which (residues 659-681) is thought to interact with the endosomal membranes and forms

channels (Oblatt-Montal et al., (1995), Protein Sci. 4, 1490-1497), are exposed on the surface of the NT. Two of the fragments of the H chain we generated and purified by chromatography are suitable for structure-function studies; the apprx 85- and apprx 45-kDa fragments beginning at residue Leu 544 and Ser 884, respectively (both extend presumably to Leu 1295) contain the channel-forming segment and receptor-binding segments, respectively. In determining partial amino acid sequences of 10 fragments, a total of 149 amino acids in the 1275-residue NT were chemically identified.

REGISTRY NUMBERS: 9001-92-7: ENDOPROTEINASE; 9004-07-3: ALPHA-CHYMOTRYPSIN
DESCRIPTORS:
MAJOR CONCEPTS: Enzymology--Biochemistry and Molecular Biophysics; Toxicology
BIOSYSTEMATIC NAMES: Endospore-forming Gram-Positives--Eubacteria, Bacteria, Microorganisms
ORGANISMS: endospore-forming gram-positive rods and cocci (Endospore-forming Gram-Positives); Clostridium **botulinum** (Endospore-forming Gram-Positives)
COMMON TAXONOMIC TERMS: Bacteria; Eubacteria; Microorganisms
CHEMICALS & BIOCHEMICALS: ENDOPROTEINASE; ALPHA-CHYMOTRYPSIN
MISCELLANEOUS TERMS: ALPHA-CHYMOTRYPSIN; **BOTULINUM** NEUROTOXIN TYPE A; CLEAVED SITE REDUCTION; ENDOPROTEINASE GLU-C; ENZYMOLOGY; FRAGMENTS; IDENTIFICATION; LIMITED PROTEOLYSIS
CONCEPT CODES:
10064 Biochemistry studies - Proteins, peptides and amino acids
10808 Enzymes - Physiological studies
22501 Toxicology - General and methods
BIOSYSTEMATIC CODES:
07810 Endospore-forming Gram-Positives

6/9/4 (Item 4 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

0011067871 BIOSIS NO.: 199799701931
Predicting differential antigen **-antibody contact regions based on solvent accessibility**
AUTHOR: Lebeda Frank J (Reprint); Olson Mark A
AUTHOR ADDRESS: Dep. Cell Biol. Biochem., Toxinology Div., U.S. Army Med. Res. Inst. Infectious Diseases, Frederick, MD 21702-5011, USA**USA
JOURNAL: Journal of Protein Chemistry 16 (6): p607-618 1997 1997
ISSN: 0277-8033
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: A novel computational approach was examined for predicting **epitopes** from primary structures of the seven immunologically distinct **botulinum** neurotoxins (BoNT/A-G) and tetanus toxin (TeTX). An artificial neural network (Rost and Sander (1994), Proteins 20, 216) was used to estimate residue solvent accessibilities in multiple aligned sequences. A similar network trained to predict secondary structures was also used to examine this protein family, whose tertiary fold is presently unknown. The algorithm was validated by showing that it was 80% accurate in determining the secondary structure of avian egg-white lysozyme and that it correctly identified highly solvent-exposed residues that correspond to the major contact regions of lysozyme-antibody cocrystals. When sequences of the **heavy** (H) **chains** of TeTX and BoNT/A-G were analyzed, this algorithm predicted that the most highly exposed regions were clustered at the sequentially nonconserved N- and **C - termini** (Lebeda and Olson (1994), Proteins 20, 293). The secondary structures and the remaining highly solvent-accessible regions were, in contrast, predicted to be conserved. In experiments reported by others, H-chain fragments that induced immunological protection against BoNT/A overlap with these predicted most highly exposed regions. It is also known that the **C - terminal** halves of the TeTX and BoNT/A H-chains interfere with holotoxin binding to ectoacceptors on nerve endings. Thus, the present results

provide a theoretical framework for predicting the sites that could assist in the development of genetically engineered vaccines and that could interact with neurally located toxin ectoacceptors. Finally, because the most highly solvent-exposed regions were not well conserved, it is hypothesized that nonconserved, potential contact sites partially account for the existence of different dominant binding regions for type-specific neutralizing antibodies.

DESCRIPTORS:
MAJOR CONCEPTS: Biochemistry and Molecular Biophysics; Immune System--Chemical Coordination and Homeostasis; Pharmacology
MISCELLANEOUS TERMS: **ANTIGEN** -ANTIBODY CONTACT REGIONS; ARTIFICIAL NEURAL NETWORK ALGORITHM; BIOCHEMISTRY AND BIOPHYSICS; **BOTULINUM** NEUROTOXIN; IMMUNE SYSTEM; NEUTRALIZING ANTIBODY; SECONDARY STRUCTURE; TYPE-SPECIFIC ANTIBODY; VACCINE DESIGN
CONCEPT CODES:
10064 Biochemistry studies - Proteins, peptides and amino acids
10506 Biophysics - Molecular properties and macromolecules
22018 Pharmacology - Immunological processes and allergy
34504 Immunology - Bacterial, viral and fungal

6/9/5 (Item 5 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

0011057448 BIOSIS NO.: 199799691508
Localization of the regions on the C - terminal **domain of the** heavy chain **of** botulinum **toxin A recognized by T lymphocytes and by antibodies after immunization of mice with pentavalent toxoid**
AUTHOR: Rosenberg Jana S; Middlebrook John L; Atassi M Zouhair (Reprint)
AUTHOR ADDRESS: Dep. Biochem, Baylor Coll. Med., One Baylor Plaza, Houston, TX 77030, USA**USA
JOURNAL: Immunological Investigations 26 (4): p491-504 1997 1997
ISSN: 0882-0139
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: We have **mapped** the regions recognized by T and/or B cells (Abs) on the **C - terminal** domain (Hc) of the **heavy chain** of **botulinum** neurotoxin serotype A (BoNT/A) after immunization of two inbred mouse strains with pentavalent toxoid (BoNTs A, B, C, D and E). Using a set of synthetic overlapping peptides, encompassing the entire Hc domain (residues 855-1296), we demonstrated that T cells of Balb/c (H-2d) mice, primed with one injection of toxoid, recognized two major regions within residues 897-915 and 939-957. After multiple inoculations with toxoid, T cells of Balb/c expanded their recognition ability and responded very well to challenge with peptide 1261-1279 and moderately to stimulation with peptide 1149-1167. Unlike Balb/c T cells, those of toxoid-primed SJL (H-2s) mice exhibited a more complex profile and responded to challenge with a large number of overlapping peptides. After one toxoid injection, however, three peptides, 897-915, 939-957/953-971 overlap and 1051-1069, were the most potent T cells stimulators. After three toxoid injections, peptides 897-915 and 1051-1069 remained immunodominant while the third region was shifted upstream to 925-9431939-957 overlap. The immunodominant **epitope** within peptide 897-915 was recognized exclusively by T cells, since no Abs were detected against this region. The Ab binding profiles of the two mouse strains were quite similar, showing only small quantitative differences. Both, Balb/c and SJL anti-toxoid Abs displayed strong binding mainly to peptide 1177-1195, followed by peptides 869-887/883-901 overlap and 1275-1296. In addition, a significant amount of Balb/c anti-toxoid Abs was bound to peptide 1135-1153. Unlike Balb/c Abs, that interacted weakly with peptides 995-1013 and 1051-1069, the anti-toxoid Abs of SJL mice exhibited strong binding toward both peptides. The results showed that, in a given strain, the regions recognized by anti-toxoid Abs and T cells may coincide or may be uniquely B or T cell determinants.
DESCRIPTORS:

MAJOR CONCEPTS: Biochemistry and Molecular Biophysics; Blood and Lymphatics--Transport and Circulation; Immune System--Chemical Coordination and Homeostasis; Infection; Metabolism; Physiology; Toxicology
BIOSYSTEMATIC NAMES: Endospore-forming Gram-Positives--Eubacteria, Bacteria, Microorganisms; Muridae--Rodentia, Mammalia, Vertebrata, Chordata, Animalia
ORGANISMS: endospore-forming gram-positive rods and cocci (Endospore-forming Gram-Positives); Clostridium **botulinum** (Endospore-forming Gram-Positives); mouse (Muridae
COMMON TAXONOMIC TERMS: Bacteria; Eubacteria; Microorganisms; Animals; Chordates; Mammals; Nonhuman Vertebrates; Nonhuman Mammals; Rodents; Vertebrates
MISCELLANEOUS TERMS: ANTI-TOXOID ANTIBODY; ANTIBODY RECOGNITION; BACTERIAL DISEASE; BIOCHEMISTRY AND BIOPHYSICS; BLOOD AND LYMPHATICS; **BOTULINUM** TOXIN A; BOTULISM; **HEAVY CHAIN CARBOXY** -TERMINAL DOMAIN ; IMMUNE SYSTEM; IMMUNIZATION; INFECTION; PENTAVALENT TOXOID; STRAIN-BALB/C; STRAIN-SJL; T-CELL; T-LYMPHOCYTE RECOGNITION; TOXICITY; TOXICOLOGY
CONCEPT CODES:
10054 Biochemistry methods - Proteins, peptides and amino acids
10058 Biochemistry methods - Carbohydrates
10064 Biochemistry studies - Proteins, peptides and amino acids
10066 Biochemistry studies - Lipids
10068 Biochemistry studies - Carbohydrates
10506 Biophysics - Molecular properties and macromolecules
13004 Metabolism - Carbohydrates
13012 Metabolism - Proteins, peptides and amino acids
15008 Blood - Lymphatic tissue and reticuloendothelial system
22501 Toxicology - General and methods
31000 Physiology and biochemistry of bacteria
34502 Immunology - General and methods
34504 Immunology - Bacterial, viral and fungal
36002 Medical and clinical microbiology - Bacteriology
BIOSYSTEMATIC CODES:
07810 Endospore-forming Gram-Positives
86375 Muridae

6/9/6 (Item 6 from file: 5)

0010885553 BIOSIS NO.: 199799519613
Epitope **regions in the** heavy chain **of Clostridium** botulinum **type E neurotoxin recognized by** monoclonal **antibodies**
AUTHOR: Kubota Toru; Watanabe Toshihiro; Yokosawa Noriko; Tsuzuki Kayo; Indoh Tomokazu; Moriishi Kohji; Saneda Kenji; Maki Yoshiyuki; Inoue Katuhiro; Fujii Nobuhiro (Reprint)
AUTHOR ADDRESS: Dep. Microbiol., Sapporo Med. Univ. Sch. Med., South 1 West 17, Chuou-ku, Sapporo 060, Hokkaido, Japan**Japan
JOURNAL: Applied and Environmental Microbiology 63 (4): p1214-1218 1997
1997
ISSN: 0099-2240
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: Seventeen **monoclonal** antibodies (MAbs) were previously established against the **heavy chain** (Hc) of **botulinum** type E neurotoxin in BALB/c mice immunized with the type E toxoid. Five MAbs (LE15-5, LE34-6, EK19-7, EK21-4, and AE27-9) showed toxin-neutralizing activity in mice. Two of the five MAbs, EK19-7 and EK21-4, recognized the regions located at amino acid positions 731 to 787 and 811 to 897, respectively. One of the remaining three antibodies (LE34-6) reacted with the amino acid sequence VIKAIN, at amino acid positions 663 to 668, closed by the ion channel-forming domain. It is suggested that the ion channel-forming domain may also be associated with the blocking of acetylcholine release. Furthermore, the amino acid sequence YLTHMRD

within 30 residues of the **C - terminal** region of the Hc component seemed to be recognized by LE15-5. It has been reported that the binding domain of the type E toxin is located on the **C - terminal** half of the Hc component. Therefore, the neutralizing activity of LE15-5 antibody may be attributed to its ability to block the binding of neurotoxin to the receptor of target cells.

REGISTRY NUMBERS: 51-84-3: ACETYLCHOLINE
DESCRIPTORS:
MAJOR CONCEPTS: Biochemistry and Molecular Biophysics; Blood and Lymphatics--Transport and Circulation; Immune System--Chemical Coordination and Homeostasis; Infection; Nervous System--Neural Coordination; Toxicology
BIOSYSTEMATIC NAMES: Endospore-forming Gram-Positives--Eubacteria, Bacteria, Microorganisms; Muridae--Rodentia, Mammalia, Vertebrata, Chordata, Animalia
ORGANISMS: endospore-forming gram-positive rods and cocci (Endospore-forming Gram-Positives); Clostridium **botulinum** (Endospore-forming Gram-Positives); Balb/C mouse (Muridae
COMMON TAXONOMIC TERMS: Bacteria; Eubacteria; Microorganisms; Animals; Chordates; Mammals; Nonhuman Vertebrates; Nonhuman Mammals; Rodents; Vertebrates
CHEMICALS & BIOCHEMICALS: ACETYLCHOLINE
MISCELLANEOUS TERMS: ACETYLCHOLINE; ACETYLCHOLINE RELEASE; AMINO ACIDS; BACTERIAL TOXINS; BIOCHEMISTRY AND BIOPHYSICS; **BOTULINUM** TYPE E NEUROTOXIN; **HEAVY CHAIN EPITOPE** REGIONS; ION CHANNEL-FORMING DOMAIN; LE15-5 ANTIBODY; **MONOCLONAL** ANTIBODIES; NEUTRALIZING ACTIVITY ; STRAIN-IWANAI; TOXICOLOGY; TYPE E; TYPE E TOXOID
CONCEPT CODES:
10010 Comparative biochemistry
10054 Biochemistry methods - Proteins, peptides and amino acids
10064 Biochemistry studies - Proteins, peptides and amino acids
10068 Biochemistry studies - Carbohydrates
10506 Biophysics - Molecular properties and macromolecules
15002 Blood - Blood and lymph studies
20506 Nervous system - Pathology
22501 Toxicology - General and methods
34502 Immunology - General and methods
36002 Medical and clinical microbiology - Bacteriology
BIOSYSTEMATIC CODES:
07810 Endospore-forming Gram-Positives
86375 Muridae

6/9/7 (Item 7 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

0008393753 BIOSIS NO.: 199294095594
MOLECULAR CLONING OF THE CLOSTRIDIUM- BOTULINUM **STRUCTURAL GENE ENCODING THE TYPE B NEUROTOXIN AND DETERMINATION OF ITS ENTIRE NUCLEOTIDE SEQUENCE**
AUTHOR: WHELAN S M (Reprint); ELMORE M J; BODSWORTH N J; BREHM J K; ATKINSON T; MINTON N P
AUTHOR ADDRESS: MOL GENETICS GROUP, DIV BIOTECHNOL, PHLS CENTRE APPLIED MICROBIOL RES, PORTON DOWN, SALISBURY, WILTSHIRE SP4 0JG, UK**UK
JOURNAL: Applied and Environmental Microbiology 58 (8): p2345-2354 1992
ISSN: 0099-2240
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: ENGLISH

ABSTRACT: DNA fragments derived from the Clostridium **botulinum** type A neurotoxin (BoNT/A) gene (botA) were used in DNA-DNA hybridization reactions to derive a restriction **map** of the region of the C. **botulinum** type B strain Danish chromosome encoding botB. As the one probe encoded part of the BoNT/A **heavy** (H) **chain** and the other encoded part of the light (L) chain, the position and orientation of botB relative to this **map** were established. The temperature at which hybridization occurred indicated that a higher degree of DNA homology

occurred between the two genes in the H-chain-encoding region. By using the derived restriction **map** data, a 2.1-kb Bg/II-XbaI fragment encoding the entire BoNT/B L chain and 108 amino acids of the H chain was cloned and characterized by nucleotide sequencing. A contiguous 1.8-kb XbaI fragment encoding a further 623 amino acids of the H chain was also cloned. The 3' end of the gene was obtained by cloning a 1.6-kb fragment amplified from genomic DNA by inverse polymerase chain reaction. Translation of the nucleotide sequence derived from all three clones demonstrated that BoNT/B was composed of 1,291 amino acids. Comparative alignment of its sequence with all currently characterized BoNTs (A, C, D, and E) and tetanus toxin (TeTx) showed that a wide variation in percent homology occurred dependent on which component of the dichain was compared. Thus, the L chain of BoNT/B exhibits the greatest degree of homology (50% identity) with the TeTx L chain, whereas its H chain is most homologous (48% identity) with the BoNT/A H chain. Overall, the six neurotoxins were shown to be composed of highly conserved amino acid domains interceded with amino acids tracts exhibiting and 110 of 845 amino acids ware conserved between H chains. Conservation of Trp residues (one in the L chain and nine in the H chain) was particuarly striking. The most divergent region corresponds to the extreme **carboxy** terminus of each toxin, which may reflect differences in specificity of binding to neurone acceptor sites.

REGISTRY NUMBERS: 142552-17-8: M81186
DESCRIPTORS: BOTB GENE HOMOLOGY L CHAIN H CHAIN AMINO ACID SEQUENCE
MOLECULAR SEQUENCE DATA GENBANK-M81186 EMBL-M81186
DESCRIPTORS:
  MAJOR CONCEPTS: Biochemistry and Molecular Biophysics; Genetics; Infection; Toxicology
  BIOSYSTEMATIC NAMES: Endospore-forming Gram-Positives--Eubacteria, Bacteria, Microorganisms
  COMMON TAXONOMIC TERMS: Bacteria; Eubacteria; Microorganisms
MOLECULAR SEQUENCE DATABANK NUMBER: M81186--GENBANK
CONCEPT CODES:
  10010 Comparative biochemistry
  10062 Biochemistry studies - Nucleic acids, purines and pyrimidines
  10064 Biochemistry studies - Proteins, peptides and amino acids
  10506 Biophysics - Molecular properties and macromolecules
  22501 Toxicology - General and methods
  31500 Genetics of bacteria and viruses
  36002 Medical and clinical microbiology - Bacteriology
BIOSYSTEMATIC CODES:
  07810 Endospore-forming Gram-Positives

6/9/8 (Item 8 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

0007678743 BIOSIS NO.: 199191061634
IMMUNOLOGICAL CHARACTERIZATION OF CLOSTRIDIUM-BUTYRICUM NEUROTOXIN AND ITS TRYPSIN-INDUCED FRAGMENT BY USE OF MONOCLONAL **ANTIBODIES AGAINST CLOSTRIDIUM-** BOTULINUM **TYPE E NEUROTOXIN**
AUTHOR: KOZAKI S (Reprint); ONIMARU J; KAMATA Y; SAKAGUCHI G
AUTHOR ADDRESS: DEP VET SCI, COLL AGRIC, UNIV OSAKA PREFECTURE, SAKAI-SHI, OSAKA 591, JPN**JAPAN
JOURNAL: Infection and Immunity 59 (1): p457-459 1991
ISSN: 0019-9567
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: ENGLISH

ABSTRACT: We examined the reactivities of Clostridium butyricum neurotoxin to nine **monoclonal** antibodies against Clostridium **botulinum** type E neurotoxin which recognize the light chain or the amino-terminal half (H-1 fragment) or the **carboxyl** -terminal half (H-2 fragment) of the **heavy chain** of **botulinum** neurotoxin. Butyricum neurotoxin and its derived chains did not react to two of four **monoclonal** antibodies recognizing the light chain, one of three recognizing the H-1 fragment,

and one of two recognizing the H-2 fragment. The results indicate that the immunological difference between the two neurotoxins is not attributable to a particular portion of the toxin molecule. The fragment of butyricum neurotoxin obtained by prolonged tryptic treatment was found to comprise the light chain and H-1 fragment linked together by a disulfide bond.

REGISTRY NUMBERS: 9002-07-7: TRYPSIN
DESCRIPTORS:
MAJOR CONCEPTS: Immune System--Chemical Coordination and Homeostasis; Infection; Toxicology
BIOSYSTEMATIC NAMES: Endospore-forming Gram-Positives--Eubacteria, Bacteria, Microorganisms
COMMON TAXONOMIC TERMS: Bacteria; Eubacteria; Microorganisms
CHEMICALS & BIOCHEMICALS: TRYPSIN
CONCEPT CODES:
10064 Biochemistry studies - Proteins, peptides and amino acids
10804 Enzymes - Methods
22501 Toxicology - General and methods
34502 Immunology - General and methods
34504 Immunology - Bacterial, viral and fungal
36002 Medical and clinical microbiology - Bacteriology
BIOSYSTEMATIC CODES:
07810 Endospore-forming Gram-Positives

6/9/9 (Item 9 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

0005211033 BIOSIS NO.: 198682057420
THE USE OF MONOCLONAL **ANTIBODIES TO ANALYZE THE STRUCTURE OF CLOSTRIDIUM-** BOTULINUM **TYPE E DERIVATIVE TOXIN**
AUTHOR: KOZAKI S (Reprint); KAMATA Y; NAGAI T; OGASAWARA J; SAKAGUCHI G
AUTHOR ADDRESS: DEP VETERINARY SCI, COLL AGRICULTURE, UNIV OSAKA PREFECTURE, SAKAI-SHI, OSAKA 591, JPN**JAPAN
JOURNAL: Infection and Immunity 52 (3): p786-791 1986
ISSN: 0019-9567
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: ENGLISH

ABSTRACT: Six **monoclonal** antibodies against Clostridium **botulinum** type E derivative toxin were prepared. Three of the five binding to the **heavy chain** neutralized the derivative toxin; the other one binding to the light chain did not. Immunoblotting analysis with the **monoclonal** antibodies showed that the fragment obtained by tryptic digestion consisted of the light chain and part of the **heavy chain** (H-1 fragment) linked together by a disulfide bond(s) and that the **antigenic** determinants common between type E and F derivative toxins were located on both the **heavy** and light **chains** . The fragment induced by chymotrypsin treatment, like the tryptic fragment, bound to four **monoclonal** antibodies. The mild tryptic treatment and reduction resulted in separation of the chymotryptic fragment into two smaller fragments corresponding to the light chain and H-1 fragment. These results indicate that H-1 fragment contains the amino-terminal portion of the **heavy chain** . The **monoclonal** antibody neutralizing the toxin and probably recognizing the **epitope** on the **carboxyl** -terminal portion (H-2 fragment) of the **heavy chain** effectively competed for binding of 125I-labeled derivative toxin to synaptosomes. Of the two **monoclonal** antibodies neutralizing the toxin and recognizing the **epitopes** on H-1 fragment, one partially inhibited binding, but the other did not. This suggests that the binding of 125I-labeled derivative toxin depends mainly on the **carboxyl** -terminal region of the **heavy chain** and that interference with binding is not the only means of toxin neutralization.

DESCRIPTORS: TOXIN NEUTRALIZATION **HEAVY CHAIN** LIGHT **CHAIN**
DESCRIPTORS:
MAJOR CONCEPTS: Immune System--Chemical Coordination and Homeostasis;

Infection; Physiology; Toxicology
BIOSYSTEMATIC NAMES: Endospore-forming Gram-Positives--Eubacteria, Bacteria, Microorganisms
COMMON TAXONOMIC TERMS: Bacteria; Eubacteria; Microorganisms
CONCEPT CODES:
06504 Radiation biology - Radiation and isotope techniques
10064 Biochemistry studies - Proteins, peptides and amino acids
10068 Biochemistry studies - Carbohydrates
10804 Enzymes - Methods
22501 Toxicology - General and methods
31000 Physiology and biochemistry of bacteria
34502 Immunology - General and methods
34504 Immunology - Bacterial, viral and fungal
36002 Medical and clinical microbiology - Bacteriology
BIOSYSTEMATIC CODES:
07810 Endospore-forming Gram-Positives

6/9/10 (Item 1 from file: 34)
DIALOG(R)File 34:SciSearch(R) Cited Ref Sci

07991491 Genuine Article#: 233UP Number of References: 52
Title: Sequence homology and structural analysis of the clostridial neurotoxins
Author(s): Lacy DB; Stevens RC (REPRINT)
Corporate Source: SCRIPPS CLIN & RES INST,DEPT MOL BIOL, 10550 N TORREY PINES RD/LA JOLLA//CA/92037 (REPRINT); SCRIPPS CLIN & RES INST,DEPT MOL BIOL/LA JOLLA//CA/92037; UNIV CALIF BERKELEY,DEPT CHEM/BERKELEY//CA/94720
Journal: JOURNAL OF MOLECULAR BIOLOGY, 1999, V291, N5 (SEP 3), P1091-1104
ISSN: 0022-2836 Publication date: 19990903
Publisher: ACADEMIC PRESS LTD, 24-28 OVAL RD, LONDON NW1 7DX, ENGLAND
Language: English Document Type: ARTICLE
Geographic Location: USA
Subfile: CC LIFE--Current Contents, Life Sciences
Journal Subject Category: BIOCHEMISTRY & MOLECULAR BIOLOGY
Abstract: The clostridial neurotoxins (CNTs), comprised of tetanus neurotoxin (TeNT) and the seven serotypes of **botulinum** neurotoxin (BoNT A-G), specifically bind to neuronal cells and disrupt neurotransmitter release by cleaving proteins involved in synaptic vesicle membrane fusion. Ln this study, multiple CNT sequences were analyzed within the context of the 1277 residue BoNT/A crystal structure to gain insight into the events of binding, pore formation, translocation, and catalysis that are required for toxicity. A comparison of the TeNT-binding domain structure to that of BoNT/A reveals striking differences in their surface properties. Further, the solvent accessibility of a key tryptophan in the **C terminus** of the BoNT/A-binding domain refines the location of the ganglioside-binding site. Data collected from a single frozen crystal of BoNT/A are included in this study, revealing slight differences in the binding domain orientation as well as density for a previously unobserved translocation domain loop. This loop and the conservation of charged residues with structural proximity to putative pore-forming sequences lend insight into the CNT mechanism of pore formation and translocation. The sequence analysis of the catalytic domain revealed an area near the active-site likely to account for specificity differences between the CNTs. It revealed also a tertiary structure, highly conserved in primary sequence, which seems critical to catalysis but is 30 Angstrom from the active-site zinc ion. This observation, along with an analysis of the 54 residue ''belt'' from the translocation domain are discussed with respect to the mechanism of catalysis. (C) 1999 Academic Press.
Descriptors--Author Keywords: clostridial neurotoxin ; **botulinum** neurotoxin ; tetanus neurotoxin ; translocation ; X-ray crystallography
Identifiers--KeyWord Plus(R): TOXIN TYPE-A; I-125-LABELED **BOTULINUM** NEUROTOXINS; NERVE-TERMINALS; TETANUS TOXIN; **HEAVY - CHAIN** ; SEROTYPE-A; NEUROTRANSMITTER RELEASE; **MONOCLONAL** -ANTIBODIES; PATTERN-RECOGNITION; CRYSTAL-STRUCTURE

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YAMASAKI S, 1994, V91, P4688, P NATL ACAD SCI USA

6/9/11 (Item 2 from file: 34)

07855011 Genuine Article#: 216DC Number of References: 273

Title: Structure, activity, and immune (T and B cell) recognition of botulinum neurotoxins

Author(s): Atassi MZ (REPRINT) ; Oshima M
Corporate Source: BAYLOR COLL MED,DEPT BIOCHEM, 1 BAYLOR PL/HOUSTON//TX/77030 (REPRINT); BAYLOR COLL MED,DEPT MICROBIOL & IMMUNOL/HOUSTON//TX/77030

Journal: CRITICAL REVIEWS IN IMMUNOLOGY, 1999, V19, N3, P219-260
ISSN: 1040-8401 Publication date: 19990000
Publisher: BEGELL HOUSE INC, 79 MADISON AVE, SUITE 1205, NEW YORK, NY

10016-7892
Language: English Document Type: REVIEW
Geographic Location: USA
Subfile: CC LIFE--Current Contents, Life Sciences
Journal Subject Category: IMMUNOLOGY
Abstract: Botulism, which was first reported over a century ago, is caused by **botulinum** neurotoxins produced by Clostridium **botulinum** in seven immunological serotypes (A through G). The primary structures of a number of these BoNTs have been determined and are reviewed here, together with their gene structure and synthesis. The biological actions of BoNTs, which result in their ability to block neurotransmitter release have been the subject of intensive study, and in this review we discuss the binding of BoNTs to the cell surface as well as the mechanism of their intercellular action. The ability of BoNTs to block neurotransmitter release has been exploited in therapeutic applications to reduce muscle hyperactivity for the treatment of a variety of clinical conditions associated with involuntary muscle spasm and contractions. The advantages, limitations, and risks of these applications are discussed. Certain compounds provide some limited protection against BoNT. However, more effective protection has been obtained immunologically either by passive immunity (i.e., by administration of anti-BoNT Abs) or by immunization with inactivated toxin. More recently, excellent protection has been obtained by immunization with the receptor-binding region comprising the **C - terminal** (residues 860 to 1296) fragment (H-c) of the **heavy chain** of BoNT/A. Here we review the **mapping** of the **epitopes** on the H-c region of BoNT/A that are recognized by anti-BoNT/A Abs raised in horse, human, and mouse. The **epitopes** on the H-c that are recognized by anti-H-c Abs and by H-c-primed T lymphocytes were **mapped** in two mouse strains [BALB/c (H-2(d)) and SJL (H-2(s))]. The peptides, which contain Ab or T cell **epitopes** (or both) on the H-c, were used as immunogens in BALB/c and SJL mice and we identified those peptides whose Ab and/or T-cell responses cross-react with H-c. Identification of these peptides is an important first step in the intricate requirements for the design of a synthetic vaccine.
Descriptors--Author Keywords: **botulinum** neurotoxin ; synthetic peptides ; antibodies ; T-cells ; **epitopes**
Identifiers--KeyWord Plus(R): TOXIN TYPE-A; COMPREHENSIVE SYNTHETIC APPROACH; PERMEABILIZED CHROMAFFIN CELLS; PROTEIN **ANTIGENIC** STRUCTURES; NUCLEOTIDE-SEQUENCE ANALYSIS; RAT-BRAIN SYNAPTOSOMES; SPERM WHALE MYOGLOBIN; ACTIVATION IN-VITRO; AMINO-ACID-SEQUENCE; ARGENTINENSE TYPE-G
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6/9/12 (Item 3 from file: 34)

01835377 Genuine Article#: JF088 Number of References: 57

Title: MINIMAL ESSENTIAL DOMAINS SPECIFYING TOXICITY OF THE LIGHT-CHAINS OF TETANUS TOXIN AND BOTULINUM **NEUROTOXIN TYPE-A**

Author(s): KURAZONO H; MOCHIDA S; BINZ T; EISEL U; QUANZ M; GREBENSTEIN O; WERNARS K; POULAIN B; TAUC L; NIEMANN H
Corporate Source: FED RES CTR VIRUS DIS ANIM,INST MICROBIOL,PAUL EHRLICH STR 28,POB 1149/W-7400 TUBINGEN//GERMANY/; FED RES CTR VIRUS DIS ANIM,INST MICROBIOL,PAUL EHRLICH STR 28,POB 1149/W-7400 TUBINGEN//GERMANY/; RIJKSINST VOLKSGEZONDHEID MILIEUHYG/3720 BA BILTHOVEN//NETHERLANDS/; CNRS,NEUROBIOL CELLULAIRE & MOLEC LAB/F-91198 GIF SUR YVETTE//FRANCE/; TOKYO MED COLL,DEPT PHYSIOL,SHINJUKU KU/TOKYO 160//JAPAN/

Journal: JOURNAL OF BIOLOGICAL CHEMISTRY, 1992, V267, N21 (JUL 25), P 14721-14729
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Abstract: To define conserved domains within the light (L) chains of clostridial neurotoxins, we determined the sequence of **botulinum** neurotoxin type B (BoNT/B) and aligned it with those of tetanus toxin (TeTx) and BoNT/A, BoNT/Cl, BoNT/D, and BoNT/E. The L chains of BoNT/B and TeTx share 51.6% identical amino acid residues whereas the degree

of identity to other clostridial neurotoxins does not exceed 36.5%. Each of the L chains contains a conserved motif, HExxHxxH, characteristic for metalloproteases. We then generated specific 5'- and 3'-deletion mutants of the L chain genes of TeTx and BoNT/A and tested the biological properties of the gene products by microinjection of the corresponding mRNAs into identified presynaptic cholinergic neurons of the buccal ganglia of Aplysia californica. Toxicity was determined by measurement of neurotransmitter release, as detected by depression of postsynaptic responses to presynaptic stimuli (Mochida, S., Poulain, B., Eisel, U., Binz, T., Kurazono, H., Niemann, H., and Tauc, L. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 7844-7848). Our studies allow the following conclusions. 1) Residues Cys439 of TeTx and Cys430 of BoNT/A, both of which participate in the interchain disulfide bond, play no role in the toxification reaction. 2) Derivatives of TeTx that lacked either 8 amino- or 65 **carboxyl** -terminal residues are still toxic, whereas those lacking 10 amino- or 68 **carboxyl** -terminal residues are nontoxic. 3) For BoNT/A, toxicity could be demonstrated only in the presence of added nontoxic **heavy** (H) **chain** . A deletion of 8 amino-terminal or 32 **carboxyl** -terminal residues from the L chain had no effect on toxicity, whereas a removal of 10 amino-terminal or 57 **carboxyl** -terminal amino acids abolished toxicity. 4) The synergistic effect mediated by the H chain is linked to the **carboxyl** -terminal portion of the H chain, as demonstrated by injection of H(C)-specific mRNA into neurons containing the L chain. This finding suggests that the H(C) domain of the H chain becomes exposed to the cytosol during or after the putative translocation step of the L chain.

Identifiers--KeyWords Plus: MOTOR-NERVE TERMINALS; MESSENGER-RNA; CLOSTRIDIAL NEUROTOXINS; TRANSMITTER RELEASE; CHROMAFFIN CELLS; NEUROTRANSMITTER RELEASE; INHIBITS EXOCYTOSIS; NUCLEOTIDE-SEQUENCE; APLYSIA NEURONS; FORMS CHANNELS

Research Fronts: 90-5196 003 (TETANUS TOXIN; **BOTULINUM** NEUROTOXIN; PHORBOL ESTERS INDUCE NEUROTRANSMITTER RELEASE)

90-2362 002 (STA58 MAJOR **ANTIGEN** GENE; RHODOCOCCUS-FASCIANS CLONING VECTORS; ESCHERICHIA-COLI CHROMOSOME; PRECISE IDENTIFICATION)

90-3172 002 (3' UNTRANSLATED REGION CONTROL MESSENGER-RNA TRANSLATION; REGULATION OF INSULIN-LIKE GROWTH FACTOR-I GENE-EXPRESSION; FERRITIN FAMILY)

90-0824 001 (**CARBOXYL** TERMINUS OF THE SACCHAROMYCES-CEREVISIAE RAS2 PROTEIN; EUKARYOTIC CELLS; MEVALONATE PATHWAY)

90-8308 001 (AUTOPHOSPHORYLATED VIRA PROTEIN; SITE-DIRECTED MUTAGENESIS; VARIANT BINDING SEQUENCE; ESCHERICHIA-COLI K-12 GENE AROG)

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6/9/13 (Item 1 from file: 73)

10698302 EMBASE No: 2000187094

Dichain structure of botulinum **neurotoxin: Identification of cleavage sites in types C, D, and F neurotoxin molecules**

Sagane Y.; Watanabe T.; Kouguchi H.; Sunagawa H.; Inoue K.; Fujinaga Y.; Oguma K.; Ohyama T.
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DOCUMENT TYPE: Journal; Article
LANGUAGE: ENGLISH SUMMARY LANGUAGE: ENGLISH
NUMBER OF REFERENCES: 33

Botulinum neurotoxin (NT) is synthesized by Clostridium **botulinum** as about a 150-kDa single-chain polypeptide. Posttranslational modification by bacterial or exogenous proteases yielded dichain structure which formed a disulfide loop connecting a 50-kDa light chain (Lc) and 100-kDa **heavy chain** (Hc). We determined amino acid sequences around cleavage sites in the loop region of **botulinum** NTs produced by type C strain Stockholm, type D strain CB 16, and type F strain Oslo by analysis of the **C - terminal** sequence of Lc and the N-terminal sequence of Hc. Cleavage was found at one or two sites at Arg444/Ser445 and Lys449/Thr450 for type C, and Lys442/Asn443 and Arg445/Asp446 for type D, respectively. In culture fluid of mildly proteolytic strains of type C and D, therefore, NT exists as a mixture of at least three forms of nicked dichain molecules. The NT of type F proteolytic strain Oslo showed the Arg435 as a **C - terminal** residue of Lc and Ala440 as an N-terminal residue of Hc, indicating that the bacterial protease cuts twice (Arg435/Lys436 and Lys439/Ala440), with excision of four amino acid residues. The location of cleavage and number of amino acid residue excisions in the loop region could be explained by the degree of exposure of amino acid residues on the surface of the

molecule, which was predicted as surface probability from the amino acid sequence. In addition, the observed correlation may also be adapted to the cleavage sites of the other **botulinum** toxin types, A, B, E, and G.

MOLECULAR SEQUENCE NUMBER: GENBANK, M92906; GENBANK, P10844; GENBANK, P18640; GENBANK, P19321; GENBANK, Q00496; GENBANK, Q60393; GENBANK, P10845
DRUG DESCRIPTORS:
* **botulinum** toxin--drug toxicity--to; *neurotoxin--drug toxicity--to
DNA
MEDICAL DESCRIPTORS:
*neurotoxicity--etiology--et; *Clostridium **botulinum**
Clostridium; toxin analysis; amino terminal sequence; nucleotide sequence; polymerase chain reaction; polyacrylamide gel electrophoresis; **antigenicity** ; nonhuman; article
CAS REGISTRY NO.: 39386-17-9 (neurotoxin); 9007-49-2 (DNA)
SECTION HEADINGS:
004 Microbiology: Bacteriology, Mycology, Parasitology and Virology
026 Immunology, Serology and Transplantation

6/9/14 (Item 1 from file: 94)
DIALOG(R)File 94:JICST-EPlus

00714746 JICST ACCESSION NUMBER: 88A0610837 FILE SEGMENT: JICST-E
Botulinum **neurotoxin.**
OGUMA KEIJI (1); YOKOSAWA NORIKO (1); FUJII NOBUHIRO (1)
(1) Sapporo Medical College
Saishin Igaku, 1988, VOL.43,NO.6, PAGE.1262-1267, FIG.3, REF.33
JOURNAL NUMBER: Z0358AAR ISSN NO: 0370-8241 CODEN: SAIGA
UNIVERSAL DECIMAL CLASSIFICATION: 615.91:579
LANGUAGE: Japanese COUNTRY OF PUBLICATION: Japan
DOCUMENT TYPE: Journal
ARTICLE TYPE: Commentary
MEDIA TYPE: Printed Publication
ABSTRACT: Clostridium **botulinum** neurotoxins can be classified into seven types, labeled A through G, based on their **antigenicity** . These toxins inhibit the release of acetylcholine from cholinergic nerve endings. All neurotoxin molecules are similar in size(M.GAMMA., about 150,000), and are composed of two suunits designated as **heavy - chain** (M.GAMMA., 100,000) and light-chain(M.GAMMA., 50,000) components. The **heavy - chain** component is further divided into two fragments of similar size(M.GAMMA., 50,000). It is proposed that the **C - terminal** fragment of the Hc component is responsible for the binding of toxin to receptors, and that the light-chain component holds the key to toxic activity.(author abst.)
DESCRIPTORS: **botulinus** toxin; food poisoning; Clostridium **botulinum** ; amino acid sequence; activation; immunogenicity; neurotoxicity
BROADER DESCRIPTORS: exotoxin; bacterial toxin; microorganism toxin; poison ; toxic substance; matter; poisoning(disease); disease; Clostridium; Bacillaceae; endospore-forming rods and cocci; bacterium; microorganism ; primary structure; structure; sequence and arrangement; molecular structure; modification; property; toxicity
CLASSIFICATION CODE(S): GZ03030F

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DIALOG(R)File 98:General Sci Abs/Full-Text

04045916 H.W. WILSON RECORD NUMBER: BGSI99045916 (THIS IS THE FULLTEXT)
Membrane fusion and exocytosis.
Jahn, Reinhard
Sudhof, Thomas C
Annual Review of Biochemistry v. 68 (1999) p. 863-911
SPECIAL FEATURES: bibl il ISSN: 0066-4154
LANGUAGE: English
COUNTRY OF PUBLICATION: United States
RECORD TYPE: Abstract; Fulltext RECORD STATUS: New record

WORD COUNT: 20829

ABSTRACT: Membrane fusion involves the merger of two phospholipid bilayers in an aqueous environment. In artificial lipid bilayers, fusion proceeds by means of defined transition states, including hourglass-shaped intermediates in which the proximal leaflets of the fusing membranes are merged whereas the distal leaflets are separate (fusion stalk), followed by the reversible opening of small aqueous fusion pores. Fusion of biological membranes requires the action of specific fusion proteins. Best understood are the viral fusion proteins that are responsible for merging the viral with the host cell membrane during infection. These proteins undergo spontaneous and dramatic conformational changes upon activation. In the case of the paradigmatic fusion proteins of the influenza virus and of the human immunodeficiency virus, an amphiphilic fusion peptide is inserted into the target membrane. The protein then reorients itself, thus forcing the fusing membranes together and inducing lipid mixing. Fusion of intracellular membranes in eukaryotic cells involves several protein families including SNAREs, Rab proteins, and Sec1/Munc-18 related proteins (SM-proteins). SNAREs form a novel superfamily of small and mostly membrane-anchored proteins that share a common motif of about 60 amino acids (SNARE motif). SNAREs reversibly assemble into tightly packed helical bundles, the core complexes. Assembly is thought to pull the fusing membranes closely together, thus inducing fusion. SM-proteins comprise a family of soluble proteins that bind to certain types of SNAREs and prevent the formation of core complexes. Rab proteins are GTPases that undergo highly regulated GTP-GDP cycles. In their GTP form, they interact with specific proteins, the effector proteins. Recent evidence suggests that Rab proteins function in the initial membrane contact connecting the fusing membranes but are not involved in the fusion reaction itself. Reprinted by permission of the publisher.

TEXT:

Key Words SNAREs, Rab proteins, SM proteins, viral fusion proteins

AN OUTLINE OF THE PROBLEM OF CELLULAR MEMBRANE FUSION

Compartmentalization into membrane-bound organelles is one of the fundamental principles of eukaryotic cells. Cellular life and differentiation depend on keeping the integrity of the boundaries of a cell and its organelles intact at all times. However, membrane-impermeable macromolecules (membrane constituents and cargo) have to be transported from one compartment to another and in and out of cells, without compromising membrane integrity. Furthermore, organelles need to be generated continuously during growth and cell division. In order to achieve this, eukaryotic organisms have developed elaborate mechanisms that execute the budding, splitting, and fusion of organelles and whole cells without leakage of intraorganellar content or disturbance of the asymmetry of the surrounding membranes. During the last 5 years, we have witnessed an explosive growth in our understanding of the molecules that govern intracellular membrane transport, especially in the secretory pathway. Progress was primarily driven by two different approaches: the biochemical dissection of the secretory machinery of vertebrate synapses and the genetic dissection of the secretory pathway in yearsts. These approaches were complemented by the reconstitution of budding and fusion reactions in cell-free systems and by targeted genetics in mice, flies, and worms. A key event in intracellular membrane traffic is membrane fusion. A picture of fusion has emerged from recent studies that suggests a common underlying mechanism for all trafficking reactions, with specialized attributes for some pathways.

Membrane fusion implies that two phospholipid bilayers merge in an aqueous environment. Before fusion, the participating membranes are separate but must be closely opposed. During fusion, an aqueous fusion pore forms that connects the compartments on the distal sides of the merging membranes and is sealed to their proximal sides. Formation of fusion pores requires nonbilayer transition states of the fusing membranes. Then the pore expands and thereby completes the fusion reaction.

Membranes do not fuse spontaneously. In fact, the repulsive energy between two opposing phospholipid membranes in an aqueous environment is very high at atomic distances (1-2 nm). These forces need to be overcome in order to reach the metastable transition states that lead to fusion.

Biological membranes use specialized fusion proteins to achieve this goal. How these proteins manage to fuse membranes is one of the fundamental problems of cell biology. Two extreme views of fusion have been proposed that suggest that the initial fusion pore is primarily proteinaceous or lipidic. In the first view (1, 2), the fusion pore is pictured as a protein doughnut that spans the two opposing membranes and forms an oligomeric ring structure. Thus, the aqueous fusion pore would be initially lined by proteins and only subsequently invaded by phospholipids. The model implies that the nonbilayer transition states of the fusing membranes are governed by protein-lipid interactions that may be both hydrophilic and hydrophobic. The proteins would guide the lipids at the contact sites through nonbilayer structures that they could not assume in the absence of proteins. The second view regards fusion as primarily mediated by phospholipids, with the role of the proteins restricted to reducing the activation energy and spatially organizing the fusion site (3). This model assumes that the fusion pore is lined by lipids and that membrane fusion must be in accordance with the physical laws determining lipidic phases.

In the following, we discuss recent advances in the understanding of membrane fusion. These advances have mainly been driven by the identification and characterization of key proteins in both intracellular and extracellular fusion reactions. Our goal is to point out unifying themes in biological membrane fusion, to integrate our knowledge about cellular fusion proteins with the biophysics of bilayer fusion, and to describe some of the unique aspects of specific fusion reactions.

FUSION OF PHOSPHOLIPID MEMBRANES

All phospholipid membrane fusion must involve transition states in which the phospholipids are not arranged in bilayers and in which the monolayers are highly curved. Such transition states are energetically unfavorable. Among other factors, the energy required for bending a monolayer depends on the lipid composition. X-ray diffraction and NMR studies have revealed that certain membrane lipids suspended in water can assume nonbilayer configurations depending on the relative space requirement of the polar head groups and their acyl side chains reviewed by Zimmerberg et al (4) . In most membrane phospholipids, the polar head groups and hydrophobic acyl side chains (tails) have approximately equal diameters; these phospholipids can be described as cylinders (5). The low-energy configuration of such lipids is the planar bilayer. Amphiphilic lipids (e.g. lysophospholipids) with relatively large head group and small tail diameters have a different shape; their shape can be described as an inverted cone. These lipids form micelles and, when present in a phospholipid monolayer, will cause "positive" curvature of the membrane (positive being defined as the curvature on the outside of a sphere). In contrast, phospholipids with small head group and large tail diameters (e.g. phosphatidylethanolamines; free fatty acids, particularly when unsaturated; diacylglycerol) are best pictured as regular cones. They can assume inverted hexagonal (HII) phases ("inverted micelles") and will support negative curvature of a membrane when enriched in one of the monolayers (5, 6).

STALK HYPOTHESIS OF MEMBRANE FUSION

Under suitable experimental conditions, it is possible to force phospholipid membranes to fuse. After temporarily reducing the repulsive forces between two opposing bilayers (e.g. by thermal fluctuations, abstraction of hydration water, charge shielding, or input of mechanical energy), relaxation may result in the merging of the proximal monolayers instead of their re-dissociation into two separate monolayers. The result is a semitoroidal structure (Figure 1) in which the proximal monolayers are connected by a highly bent stalk, and the distal monolayers are pulled toward each other, forming a dimple (7). Transition states in which the proximal but not the distal leaflets are continuous were shown to exist in many independent studies. These transition states are often referred to as stalks or hemifusion states (8).

How does the stalk intermediate lead to the opening of a fusion pore? Originally, it was assumed that relaxation of the monolayer bending energy would result in the formation of a central, disk-shaped diaphragm consisting of a single bilayer derived from the two distal monolayers also referred to as unrestricted hemifusion, Figure 1 (7, 9) . The diaphragm of

the unrestricted hemifusion state was thought to expand until the accumulated tension destabilizes the structure, resulting in the opening of single or multiple fusion pores. In a modified version of model, the contribution of the hydrophobic void spaces (see Figure 1) was taken into account. The creation of such void spaces is energetically highly unfavorable. After contact between the two distal monolayers has been established, relaxation would result in the breakup of the connection rather than the formation of a diaphragm (10). However, any lowering of the void energy levels (e.g. by long chain alkyl side chains, isoprenoid lipids) would stabilize the transition state and thus may favor formation of a diaphragm (11). In both models, the generation of small fusion pores may be associated with a phase transition to isotropic, micellarlike "cubic" (nonlamellar) phases. Such lamellar-nonlamellar phase transitions can be observed in artificial systems under certain conditions (see e.g. 12, 13).

The stalk hypothesis is currently the only coherent concept that describes the transition states during membrane fusion. It is supported by theoretical considerations and a variety of experimental approaches, whereas alternative models (e.g. involving inverted micelles rather than curved but intact monolayers during the transition state) failed critical theoretical and experimental tests reviewed by Chernomordik (14) . For instance, the predicted sequence of events (cis-leaflet mixing, transient pore opening, trans-leaflet mixing, full pore opening) has recently been documented in a model fusion reaction (15). Furthermore, the stalk model predicts that addition of inverted cone-shaped lipids (supporting positive curvature) to the cis-sided leaflets would block stalk formation whereas their addition to the trans-leaflets would promote fusion pore opening. Correspondingly, the opposite effects would be expected when cone-shaped lipids are added. Experiments using a variety of model systems largely support these predictions (4, 13, 16). Interestingly, it has recently been observed that during fusion of a liposome with a planar lipid membrane the fusion pores undergo transient and reversible opening and closings (flickering) before dilation (17). Such flickering, originally thought to be a hallmark of protein-mediated fusion (2), is thus being recognized as a normal intermediate during the fusion of lipidic bilayers.

VIRAL FUSION PROTEINS: HOW DO THEY WORK?

Many viruses contain a membrane that encloses the viral nucleocapsid. During infection, the viral membrane fuses with the host cell membrane, thereby injecting the nucleocapsid into the cytoplasm. Some viruses, for example, influenza virus, are endocytosed and only fuse after they have reached an endocytic acidic interior. Other viruses, for example, the human immunodeficiency virus (HIV), fuse with the plasma membrane directly after binding to the host cell receptor (18). A fascinating aspect of viral infection is that in most cases, binding of the virus to the host cell membrane and fusion of the viral membrane with this membrane are carried out by a single protein referred to as the viral fusion protein. Viral fusion proteins are glycoproteins with a single transmembrane region and relatively large extraviral domains that are exposed on the surface of the virus particle. In spite of similar functions, viral fusion proteins are diverse, with little sequence similarities between various viruses.

Much is known about viral fusion proteins, although their mechanisms of action are still far from clear. Evolutionarily unrelated viral fusion proteins share structural features that appear to be responsible for their fusogenic activities and that exhibit similarities with eukaryotic fusion proteins. In the following, we focus on selected features of viral membrane fusion with particular emphasis on the structural changes involved in the fusion reaction. For more comprehensive overviews, the reader is referred to several excellent recent reviews (18-24).

FUSION PEPTIDES

Most viral fusion proteins contain a short amphiphilic peptide domain, the fusion peptide, which is essential for fusion. The fusion peptide typically encompasses 20-30 residues whose sequence is conserved within a virus family but not between different viruses (25). A common feature of fusion peptides is alternating hydrophobic and charged amino acids with a potential to form amphipathic helices. The best studied fusion peptides include those of the influenza virus hemagglutinin (HA) and of gp41 of the

HIV (22, 26-28). These peptides assume flexible random conformations in an aqueous environment and become structured upon insertion into phospholipid bilayers. The exact structure assumed by a fusion peptide inserted into a phospholipid bilayer is unclear, but at least part of the peptide forms an amphiphilic a-helix (22, 29-31). Using hydrophobic photoactived cross-linkers, Durrer and co-workers demonstrated that in the case of HA, the fusion peptide is the only sequence of the viral fusion protein that inserts into the hydrophobic interior of the bilayer during fusion (32).

It remains a challenge to clarify the precise role of the fusion peptide during viral membrane fusion. At least three functions are possible. First, the peptide may physically attach the fusion protein to the target membrane and, in doing so, provide an anchor for the exertion of force by the fusion protein onto the phospholipid bilayers. Second, by virtue of its amphiphilic nature, the fusion peptide may destabilize the membranes and thus directly promote monolayer mixing. This function implies that the fusion peptide is the active principle in lowering the activation energy for fusion. Third, the fusion peptide may form a proteinaceous fusion intermediate, for example by assembly of homomultimers that line the fusion pore.

These putative functions are not necessarily exclusive. As discussed further below for HA and gp41, it is generally believed that fusion peptides are essential for anchoring the fusion protein to the target membrane. Studies on the interactions of fusion peptides with membranes also provide support for the other two functions. Isolated fusion petides trigger fusion of liposomes, probably by destabilizing the outer monolayer of the vesicles reviewed by Durell et al (22) . Insertion of fusion peptides affects lipid side-chain packing by expanding the outer monolayer (33), causes leakage of aqueous contents (34), and favors the transition to isotropic cubic/hexagonal phases (35, 36). Isolated HA fusion peptides do not span the membrane but are oriented at an angle that is slightly titled with respect to the plane of the membrane. As a result, the fusion peptides are partially buried in the hydrophobic interior (see e.g. 29, 30, 34, 37). Fusion peptides not only bind to phospholipid bilayers but also interact with each other and with other peptides while bound to the membrane (37, 38). Since several copies of a viral fusion protein are needed in a fusion reaction, it is possible that oligomerization of fusion peptides plays a critical role in stabilizing a fusion intermediate, such as the fusion pore, after the outer leaflets of the fusing membranes become attached to each other.

It should be noted, however, that amphiphilic peptides, irrespective of whether or not they are involved in fusion, bind to bilayers, destabilize them, and even fuse them. Furthermore, membranes spontaneously fuse in vitro when exposed to any surface-active molecules, including proteins such as annexins, for which there is no evidence that fusing membranes belongs to their biological duties (39). However, addition of amphiphilic peptides to artificial membranes results in a random distribution of these peptides on the surface of all liposomes, creating a scenario quite different from what happens during the locally confined interactions between viral and target membranes. Thus, the in vitro properties of fusion peptides, although compatible with various functions in membrane fusion, cannot be considered as evidence for such functions. This evidence can clearly only be obtained by studying fusion peptides in the context of the complete viral fusion proteins.

INTERMEDIATES IN VIRAL MEMBRANE FUSION

Similar to phospholipid membrane fusion and exocytotic membrane fusion (see below), viral fusion involves the transient and reversible formation of aqueous fusion pores (40). In HA-mediated fusion, pore opening precedes lipid mixing, suggesting either that HA forms a proteinaceous fusion pore or that in a partially lipidic fusion pore the lipids in the proximal leaflet cannot diffuse freely. Such a restriction in lipid diffusion may be caused by interactions of the lipids with the fusion protein (41-44). Since membrane orientation is maintained during fusion (45), intermediate stages involving isotropic randomization of both inner and outer leaflets (HII phases) do not occur. Addition of "inverted cone" phospholipids such as lysophospholipids inhibit fusion, as predicted by the stalk theory, but at least some of these effects may be attributed to a direct interference with fusion peptide binding (46).

A key observation in the understanding of viral membrane fusion was obtained with a mutant HA in which the transmembrane region was replaced by a phosphatidylinositol-glycan anchor. This HA mutant still promoted full-scale lipid mixing but fusion pore formation was blocked (47). Thus, HA without a transmembrane region can still cause membrane attachment, but the normal formation of a fusion pore is bypassed. Instead, a stable "dead-end" state of extended hemifusion develops. Further studies by Chernomordik and colleagues (48) with suboptimal activation of HA (e.g. by lowering the pH to 5.3 instead to 4.9) extended these results. With partially activated HA, unrestricted lipid mixing with only initial and transient pore flickering was observed. Once this state was reached, it was difficult to induce fusion by transfer to optimal conditions, again suggesting an unrestricted hemifusion diaphragm. It seems likely that under normal conditions, HA proteins form a ring with a fusion pore that restricts lipid mixing until the pore starts dilating. If HA lacks a transmembrane region or is only partially activated, the HA ring cannot generate a fusion pore. Instead, inserted HA fusion peptides induce a dead-end escape reaction, which is associated with production of an unrestricted hemifusion state that is energetically stable (48).

Together these findings are consistent with the stalk hypothesis in that the proximal monolayers can merge before the fusion pore opens. However, the restriction of lipid flow during a normal fusion reaction indicates that the fusion proteins tightly control the patch of phospholipid bilayers in which fusion takes place, suggesting that protein-lipid interactions control the intermediate stages of the fusion reaction. It remains to be established whether a small hemifusion diaphragm needs to form before the fusion pore opens (47, 48) or whether pores form without a "single bilayer" intermediate in a more complex, semitoroidal structure involving cubic phases, as postulated by the modified version of the stalk theory (49).

NANOMECHANICS OF VIRAL FUSION PROTEINS

In order to fuse an enveloped virus with the host cell, the viral fusion protein must overcome the energy barrier separating the two membranes and initiate the formation of fusion intermediates. Fusion therefore requires the generation of mechanical force that brings the membranes closely together and bends the membranes as required for the initiation of fusion.

Although many uncertainties surround the mechanics of viral fusion, a few general principles begin to emerge. Recent progress is largely due to advances in the crystallization of fragments of viral fusion proteins reviewed by Skehel & Wiley (50) . The HA and HIV fusion proteins are synthesized as single transmembrane precursors that are subsequently cleaved into two subunits. The fusion protein precursor in influenza, HA0, becomes fusion competent when processed into the larger HA1 and the smaller HA2 subunit that remain linked by disulfide bonds. Three HA1/HA2 complexes assemble into trimers that project as 13-nm long spikes from the viral surface at neutral pH. In striking analogy, the unrelated HIV-1 envelope glycoprotein gp160 is proteolytically processed to yield two subunits, gp120 and gp41. The large globular domains, HA1 and gp120, are responsible for receptor recognition and binding: terminal glycoprotein/glycolipid sialic acid moieties for HA1, CD4 receptors together with chemokine coreceptors for gp120. The small subunits, HA2 and gp41, bear the transmembrane domain and contain the fusion peptide see Hernandez et al (18) and Wyatt & Sodroski (51) for review .

Key to the understanding of the influenza and HIV fusion proteins is the fact that proteolytic processing generates a metastable fold that resembles a loaded spring (52). Activation triggers relaxation of the proteins, resulting in dramatic conformational changes that expose the previously buried fusion peptides. In the activated form, the N-terminally located fusion peptides reside on top of an extended, triple-stranded coiled coil. Once activated, the fusion proteins are no more fusogenic and unusually stable (53; and references therein). Thus, the conformational changes during activation drive the fusion reaction, with the activated protein representing an end-stage condition that is no more active. Probably, the fusion protein injects the fusion peptide into the target membrane during activation and subsequently forces a reorientation of the two phospholipid bilayers. Reorientation may be mediated by an alignment of

oligomers of the fusion peptides and transmembrane regions that finally induce the formation of a fusion pore.

HA is the only viral fusion protein for which partial crystal structures are available for both the metastable (neutral) and relaxed (low pH) conformations (54-56). In the neutral form, the three fusion peptides of the trimer are buried inside the HA2 domains, where they lie in close proximity parallel to the viral membrane (57). Upon activation, a major loop connecting two antiparallel helical domains of each monomer (helix D-C with helix A, see Figure 2) is transformed into a helix (helix B), resulting in an extended triple-stranded coiled-coil, with the fusion peptides on top. In the intact protein, this change would propel the fusion peptide over the distance of 10 nm distal from the viral surface toward the target membrane. In addition, rearrangements occur at the proximal end of the helical bundle. Most notably, helix D switches orientation, resulting in an antiparallel orientation to helix C. As a result, the last **C - terminal** amino acids contained in the structure are pushed "upward" toward the fusion peptide (Figure 2). Finally, the domain connecting the protein to the transmembrane region becomes unstructured, indicating an increase in flexibility (56).

Oddly, it is still mysterious how these changes bring about fusion. Since before activation of HA the target membrane is kept 10 nm away from the viral membrane, the fusion peptide first needs to grab the host cell membrane and then pull it close to the viral membrane. Unfortunately, the position of the transmembrane region is unknown owing to the flexibility of the **C - terminal** portion. Thus, the relative positions of the fusion peptide and the transmembrane regions, i.e. the critical parts of HA that interact with the phospholipid membrane, cannot be deduced from the crystal structure (50). Both photoaffinity labeling (28) and immunoelectron microscopy (58) show that in the absence of a target membrane, the fusion peptide dives into the membrane of its resident virus, thus being localized beneath the transmembrane region. If the fusion peptide, as most researchers agree, is first inserted in the target membrane, then the helical bundle subsequently must bend or rotate. This would require a two-step refolding mechanism Figure 2; reviewed by Hughson (59) . In the first step, HA would eject its fusion peptide away from the viral surface and insert it into the target membrane. In the second step, helix D reverses and aligns itself along helix C, thereby providing the driving force for pulling the two membrane anchor domains together at the site of membrane contact "jackknife" mechanism; see Hughson (21) . In this scenario the helical bundle, attached by the fusion peptides to the target membrane, acts as a lever on which force is exerted by the assembly of helix D with the attached transmembrane domain anchored in the viral membrane.

An interesting alternative hypothesis suggests that activation triggers insertion of the fusion peptide not into the target membrane but into the viral membrane, without bending or turning of the fusion protein (60). The hypothesis suggests that the formation of the coiled-coil bundle after the insertion of the peptide pulls the peptide and the viral membrane up toward the target membrane, with reorientation occurring after initiation of fusion. This model is more satisfying in terms of nanomechanics since the formation of the stiff helical bundle would provide a scaffold for pulling the viral membrane upward, and it would be the energy released during the formation of the coiled-coil bundle that drives the reaction rather than a bending/reorientation reaction involving the presumably flexible connection to the transmembrane domain. However, the problem with this model is that subsequent fusion would have to be completely lipidic, at least on the side of the target membrane, making it hard to understand how a fusion pore could form initially without any lipid mixing.

In gp41, two large helical domains are adjacent to the N-terminally located fusion peptide. They are connected by a loop that is held together by disulfide bonds reviewed by Hernandez et al (18) . The crystal structures of protease-resistant fragments revealed that the N-terminal helical domains of the three subunits form an extended, triple-stranded coiled-coil with the amino-terminus carrying the fusion peptide at its tip. The three **carboxy** -terminal helices bind to the outside of the bundle. They pack into the three hydrophobic grooves of the coiled-coil in an antiparallel orientation, thus placing the **C** - and N- **terminal** ends to the same side of the elongated complex (61-63). The resulting six-helix

bundle is about half as long as the trimeric coiled-coil of activated HAII (61). A very similar structure has recently been reported for the related gp41 of simian immunodeficiency virus (64).

How does this structure relate to fusion activity? Presently, no atomic structure of any other conformation of gp41 is available. However, important clues were obtained from biochemical studies. Peptides corresponding to the **C - terminal** helical domains are potent and dominant inhibitors of HIV-induced membrane fusion (65, 66). Interestingly, these peptides are only effective when the protein is activated by interaction with its CD4-receptor. Most likely, these peptides bind to the grooves of the central triple-stranded coiled-coil, competing with the corresponding domains of the native protein. The efficacy of these peptide inhibitors at low concentration is surprising considering that the native helix is tethered to the bundle and thus should have an advantage in the binding reaction (see 66 for a more detailed discussion). These findings are best explained by a model that involves an intermediate conformation. This intermediate is generated during receptor contact, persists for some time, and allows the peptides to bind to the exposed grooves on the N-terminal coiled-coil see Hughson (21) and Chan & Kim (66) for review . When the protein is arrested by such peptides after activation, some lipid mixing is observed but no fusion pore is established (67), suggesting that although the initial energy barrier separating the membranes is overcome, the protein is incapacitated in its ability to mediate complete fusion. Most current models invoke a two-step mechanism of gp41 that bears striking similarities with that discussed above for HA 61; for review, see Hughson (21) and Chan & Kim (66); see Figure 2 . Upon activation, the large subunit gp120 dissociates and gp41 ejects the fusion peptide, positioning it at the tip of the extended helical bundle where it dives into the interior of the target membrane. This would be the state that is inhibited by peptide binding. Subsequently, the helices bend in the linker region connecting the **C** -and N- **terminal** helices and establish antiparallel helical contacts, resulting in a jackknife move also referred to as hairpin (66) or mousetrap (68) that effectively pulls the membranes together.

Even if many aspects of the nanomechanics involved in fusion are still speculative, it is evident that there are stunning structural and mechanistical parallels between gp41 and HA2. These parallels probably extend to additional viral fusion proteins as highlighted by the crystal structures of fusion proteins of Ebola virus (69) and Moloney murineleukemia virus (70). As we see in the following paragraphs, some of these principles also apply to fusion proteins operating in intracellular fusion in eukaryotic cells.

PRINCIPLES OF INTRACELLULAR FUSION REACTIONS

Given the vast diversity of intracellular fusion events in eukaryotes, the question arises whether these fusion reactions share features with each other and with viral and lipidic fusion reactions. Unfortunately, little is known about early transition states in intracellular fusion except for one particular type of intracellular fusion, the fusion of a secretory vesicle with the plasma membrane (exocytosis) that can be monitored with sophisticated electrophysiological and optical approaches. Analyses of exocytosis in cells with relatively large secretory organelles, such as mast cells or chromaffin cells, revealed that exocytosis involves the formation of transient and reversible fusion pores that can go through cycles of rapid openings and closings (flickering) (1, 2).

Formation of a fusion pore may allow for the transfer of lipids between the fusing secretory vesicle and the plasma membrane, demonstrating that the pore is not entirely composed of protein like a channel. Thus, exocytotic membrane fusion involves fusion pore intermediates with properties similar to those observed in viral and lipidic fusion reactions. Other intracellular fusion reactions have not yet been investigated in similar detail, but it seems likely that the properties of exocytotic fusion are paradigmatic for all cellular fusion reactions. This allows for the tentative conclusion that independent of the forces that drive and control fusion, the biophysical processes, and transition states governing the fusion of phospholipid membranes, viral membranes, and cellular membranes are similar.

UNIVERSAL COMPONENTS

Are there cellular fusion proteins that act like viral fusion proteins or is the mechanism of protein action in intracellular fusion dramatically different? Although this question cannot be answered at present, progress has recently been made in identifying obligatory components of cellular fusion events. At least three classes of proteins, discussed in detail in the following chapters, appear to be universally involved in all intracellular fusion reactions. They include the following:

1. SNARE proteins. SNAREs (soluble NSF receptors) represent a class of membrane-bound proteins that fall into two groups, Q-SNAREs and R-SNAREs. The best characterized SNAREs are the neuronal SNAREs functioning at the synapse: Synaptobrevins/VAMPs (vesicle associated membrane protein) are localized to synaptic vesicles, whereas syntaxin 1 and SNAP-25 are primarily present on the plasma membrane. SNAREs are weakly homologous to each other in a sequence motif called the SNARE motif, which mediates the assembly of SNAREs into core complexes during fusion. Core complexes are disassembled by the soluble ATPase NSF in conjunction with SNAP proteins.

2. Sec1/Munc 18 homologs (SM proteins). These are soluble proteins of [similar]65 kDa that bind to Q-SNAREs of the syntaxin family. At the synapse, syntaxins cannot simultaneously bind to SM proteins and other SNAREs, suggesting that these complexes form sequentially.

3. Rab proteins. Rab proteins are small GTPases ([similar]20-25 kDa). In yeasts, they genetically interact with SNAREs and SM proteins. Thus, the three universal components of fusion reactions are functionally connected, although the precise nature of these interactions is unclear.

CONFUSION ABOUT CELLULAR MEMBRANE FUSION: MODEL SYSTEMS AND TERMINOLOGY

Progress in our understanding of fusion proteins is largely due to the establishment of suitable model systems. Most intensely studied are the various stages of the secretory and vacuolar pathway in yeasts e.g. endoplasmic reticulum (ER) to Golgi traffic, exocytosis, vacuole maturation , the fusion of endosomal and Golgi membranes and vesicles, and synaptic vesicle exocytosis (for reviews, see e.g. 71-77). Our discussion emphasizes the mammalian synapse and the yeast secretory and vacuolar pathways for the following reasons. First, the most proteins were identified in these systems, and the most detailed analyses were made here. Second, these model systems cover evolutionary distant and kinetically diverse fusion reactions, and principles applying to both systems are therefore likely to be valid for all eukaryotic fusion reactions. The strength of the synaptic system lies in the combination of protein biochemistry with mouse genetics an d electrophysiology. The major advantage of the yeast vacuolar system lies in the exquisite genetic analysis and the availability of an in vitro assay for fusion that is easily manipulated. In both systems, identification of key proteins by biochemical characterization (mammalian synapse) or genetic screens (yeast vacuole) formed the basis for all subsequent studies. The synaptic system, however, allows measurements of fusion in real time, which is not possible in yeasts. Conversely, in the yeast system, reconstitution and in vitro methods, which are not available for the synapse, have permitted manipulations that will eventually lead to a complete reconstitution of fusion.

In spite of a convergence of fusion proteins and fusion mechanisms, there has been an unfortunate divergence in terminology with respect to terms such as docking, priming and fusion. For instance, in the in vitro assay for vacuolar fusion, priming precedes docking and involves an activation of SNAREs by dissociating SNARE complexes (78; see below for details). At the synapse, in contrast, priming follows docking and probably involves the formation of SNARE complexes. Therefore, in two different systems, the term priming means exactly the opposite, i.e. the dissociation of SNAREs in yeast vacuole fusion and the formation of SNARE complexes in the synaptic vesicle exocytosis.

What are the common steps in such fusion reactions?

1. In preparation to fusion, two events occur: SNARE proteins are activated by NSF which functions as a chaperone to dissociate SNARE core complexes, and Rab proteins are targeted to the cognate organelles and loaded with GTP. We refer to this preparatory step as fusion setup.

2. Before fusion, the two membranes need to recognize each other and become attached. Attachment is reversible. For instance, optical recordings

using total internal reflection microscopy showed that in adrenal chromaffin cells, secretory vesicles that are already firmly attached to the plasma membrane can leave again without fusing (79, 80). This step is referred to here as membrane attachment. It is conceivable that fusion setup and membrane attachment are at least partially independent of each other and may occur in reversed order in some systems.

3. After membrane attachment, fusion is initiated in a step that involves assembly of the SNARE core complex and Rab function. This step may already involve partial fusion (for instance, a stalklike hemifusion intermediate), which is a prerequisite for the formation of fusion pores. We refer to this step, which presumably includes several subreactions, as prefusion.

4. The fusion reaction is completed by merging both the proximal and the distal leaflets of the fusing membranes to open the fusion pore. We refer to this step as fusion pore formation.

5. Finally, the fusion pore expands in a step that we call fusion pore dilation.

In the following, we use these terms as defined and refer to the entire overall process as fusion. We hope that by using neutral terms, we can avoid the semantic confusion that imperils a balanced discussion, for example if docking involves SNAREs or not.

STRUCTURE OF SNARES AND THE CORE COMPLEX

SNARE PROTEINS FORM A SUPERFAMILY

SNARE proteins were independently discovered in yeast cells and neurons reviewed by Bennett & Scheller (81) and Ferro-Novick & Jahn (82) . On the basis of their localization and overall structure, SNAREs were initially classified into t-SNAREs (for SNAREs localized to the target membrane) and v-SNAREs (for SNAREs localized to the membrane of the trafficking vesicle) (83). Following the synaptic paradigm, the t-SNAREs were grouped into the syntaxin and SNAP-25 families, whereas the v-SNAREs comprised synaptobrevin/VAMPs and their relatives. In the last few years, many additional SNARE proteins have been discovered. Some of these are only distantly related to the three neuronal "founding members" of the v- and t-SNARE families and cannot be easily assigned to one of the synaptic groups. However, sophisticated profile-based sequence analyses revealed that all SNAREs share a homologous domain of [similar]60 amino acids that is referred to as the SNARE motif (84-86). The SNARE motif is the defining feature of all SNAREs and is also functionally important because it mediates the association SNAREs into core complexes (see below). In addition to SNARE motifs, SNAREs contain flanking sequences that attach them to membranes and mediate additional protein-protein interactions or both.

SNAREs can be divided into subfamilies on the basis of whether they contain one or two SNARE motifs, on the sequences of the SNARE motifs (85), and on the type and sequences of the flanking domains. Most SNAREs contain a single SNARE motif that is preceded by a variable N-terminal sequence and is followed by a **C - terminal** transmembrane region. This SNARE group comprises the syntaxin and synaptobrevin subfamilies as well as Bet1p, Bos1p, and their relatives. Other SNAREs do not have transmembrane domains but are membrane anchored by post-translationally attached lipids. For example, SNAP-25 and its relatives contain two SNARE motifs that are separated by a cysteine-rich sequence. The cysteines are palmitoylated and thereby attach SNAP-25 to the membrane (87). In addition, some SNAREs without a transmembrane region contain only a single SNARE motif, for example Vam7p (85, 88) and syntaxin variants with cysteine residues at the C - **terminal** end (89, 90). These SNAREs are presumably lipid-modified at the **C - terminus** . Finally, a protein called tomosyn probably represents an unusual SNARE that may be involved in controlling syntaxin. Tomosyn has a large multidomain structure and binds to syntaxin via a **C - terminal** SNARE motif related to synaptobrevin (91).

SNAREs also differ in the sequences that surround the SNARE motifs or the membrane attachment domains. In many SNAREs, the SNARE motifs and membrane attachment domains are flanked by only short additional sequences that do not form independently folding domains, such as the N-terminal proline-rich sequence of most synaptobrevins/VAMPs. In contrast, the N-termini of syntaxins are composed of separate domains. These domains are conserved between syntaxins that function at the same trafficking step but

differ between syntaxins that function at distinct trafficking steps, while the SNARE motifs of all syntaxins are homologous (85). This architecture of syntaxins indicates that their N-terminal domains are specific for a given trafficking step, whereas their SNARE motifs are more promiscuous.

Table 1 summarizes the SNARE proteins that were known at the time that this chapter was finished. In Table 1, SNAREs are classified into Q- and R-SNAREs because the crystal structure showed that a central residue in the SNARE motif is either an arginine or a glutamine. These central arginine and glutamines form an ionic layer in which three Q-SNAREs bind to one R-SNARE (see below; 92). The classification into Q- and R-SNAREs is preferable over the v- and t-SNARE terminology because, as discussed in more detail below, localization on trafficking vesicles vs. target membranes does not always correlate with structurally identified SNARE subfamilies (93).

How many different SNAREs do exist in a given organism? Although most authors agree that all syntaxinlike Q-SNAREs present in the yeast genome are identified (eight total; 94), the presence of additional R-SNAREs and of more distant members of the family cannot be ruled out. In plants and mammals, new SNAREs are being characterized at a rapid pace, mostly owing to the growth of the sequence databases and increasingly sophisticated search algorithms. Therefore, the SNARE superfamily is likely to have many more members than are known today.

SNARE MOTIFS FORM CORE COMPLEXES

The neuronal SNAREs synaptobrevin 2, syntaxin 1a, and SNAP-25 assemble into a stable ternary complex with a 1:1:1 stoichiometry that is referred to as the core complex. The ternary complex is unusually stable. Under laboratory conditions, assembly is virtually irreversible. The complex is not denatured by heat (95) or by the detergent SDS (96), and it is resistant to proteolysis by **botulinum** and tetanus neurotoxins (96). Resistance to SDS and neurotoxins is therefore often used to demonstrate the presence of ternary core complexes. Site-directed mutagenesis and limited proteolysis revealed that synaptobrevin, syntaxin, and SNAP-25 bind to each other exclusively via their SNARE motifs (96-103). A core complex formed by the isolated four SNARE motifs without surrounding sequences (one SNARE motif each from syntaxin and synaptobrevin and two from SNAP-25) exhibits most of the biophysical and biochemical properties of complexes formed by the intact proteins (103). CD-spectroscopy and site-specific labeling showed that the core complex is composed of a helical bundle with the transmembrane regions emerging from the **C - terminal** end of the rod-shaped particle (95, 101, 103-105). The N-terminal domain of syntaxin emerges from this particle as a mobile, separately folded domain (104, 106). Although not yet studied in similar detail, the exocytotic fusion complex from yeasts (consisting of corresponding fragments of Sec9p, Snc1p, and Sso1p) exhibits very similar properties (107, 108), suggesting that the features of the neuronal SNARE complex are paradigmatic for all SNARE complexes.

The crystal structure of the synaptic core complex has recently been solved (92). It consists of a twisted four-helical bundle with an overall length of 12 nm. All chains are aligned in parallel, a finding that was independently confirmed by site-specific labeling (109, 110). The linker domain of SNAP-25, which connects the two helical SNARE motifs and contains the cysteine-rich membrane anchor domain, is not part of the crystal structure; it probably forms a loop that connects the two SNARE motifs of the molecule (see Figure 3). Interactions in the core of the bundle are mostly hydrophobic, resembling that of other helix bundles with coiled-coil structures. Interestingly, an ionic layer is formed in the center of the four-helical bundle. This ionic layer is constructed from an arginine residue contributed by the SNARE motif of synaptobrevin and three glutamine residues contributed by each of the three SNARE motifs of syntaxin and SNAP-25, respectively. Together with the peptide backbones, the flanking leucine-zipper layers form a water-tight seal around the ionic layer, thereby shielding the ionic interactions from the aqueous surroundings that may increase the stability of the complex. Furthermore, the asymmetric ionic layer fixes the positions of the hydrophobic layers in the center of the helix bundle. This ensures that the long helices of the SNARE motifs are placed into the correct "register" during assembly (92).

The amino acids contributing to the ionic layer are the most highly

conserved residues throughout the SNARE superfamily (86). Apparently, SNARE core complexes generally consist of four-helix bundles, formed from one R-SNARE and three Q-SNAREs with an ionic layer sandwiched between hydrophobic layers, although complexes consisting of only Q-SNAREs have been invoked in special cases (see below). The functional significance of the four-helix bundle is highlighted by the phenotypes of mutations in SNARE proteins in various organisms. Single amino acid substitutions **mapping** to the core of the bundle generally result in loss, or at least severe impairment, of SNARE function (93).

The surface of the complex includes four shallow grooves that contain patches of charged and hydrophobic regions. It is possible that this surface provides a scaffold for the binding of regulatory proteins. The amino acids exposed on the surface are the least conserved residues (93). Hydrogen-bonding and surface electrostatic interactions further stabilize the helix bundle. Interestingly, there are significantly fewer of such interactions originating from synaptobrevin than from the other three helices (92).

ASSEMBLY OF SNARE CORE COMPLEXES

The essential nature of SNARE core complexes in membrane fusion and their unusual biophysical properties (stability, energetic state) have stimulated extensive studies of their formation, primarily with the synaptic SNAREs. From these studies, several principles are beginning to emerge.

First, SNAREs readily assemble into core complexes whenever they are solubilized in nondenaturing detergents (111). This feature complicates studies assessing the assembly status and composition of SNARE complexes in living cells or intact membranes. It is likely that in vivo at any given time, only a fraction of SNAREs are assembled into complexes, but analysis of the exact degree of assembly and its regulation has proved problematic.

Second, although truncated and/or partial complexes can be generated, they are less stable than the ternary complex (96, 98). Binary complexes of synaptobrevin with syntaxin or SNAP-25 have little stability (95), probably because of the low number of side-chain interactions with the other two proteins (92). The more stable syntaxin--SNAP-25 complex has a 2:1 stoichiometry (95), possibly forming a four-helix bundle with only glutamines in the "ionic" layer. It is unclear if this binary complex is ever present in vivo. When synaptobrevin is added, one of the syntaxin molecules is displaced (95). In addition, unstable complexes can be formed from fragments containing N- or **C - terminal** deletions in the SNARE motif, such as those generated by cleavage with **botulinum** neurotoxins (96, 99, 112, 113). The formation of such shortened complexes is not surprising when considering the multiplicity of interchain contacts in the long helical bundle.

Third, the SNARE motifs undergo major conformational changes during assembly. The SNARE motifs of syntaxin, SNAP-25, and synaptobrevin are unstructured when they are not assembled into core complexes but roll up into helices upon assembly (95, 114) (J Rizo, personal communication). Very similar changes were observed with the corresponding yeast proteins (108, 115).

Fourth, core complex formation appears to be a promiscuous property of SNAREs with little specificity. Several yeast SNAREs have been shown to form multiple core complexes in vivo, and many SNAREs bind to each other in vitro after tissue solubilization. Furthermore, recombinant purified SNAREs can be made to bind to each other in vitro in almost any combination, although the stability of the complexes greatly varies. These findings suggest that core complex formation does not confer specificity onto membrane interactions.

Fifth, the transmembrane domains of synaptobrevin and syntaxin form homo- and heterooligomeric complexes (116, 117) and thus contribute to the overall stability of the complex (101). Furthermore, these domains may cause association of ternary complexes into larger entities that may play a role during membrane fusion. Again, it is unclear if these complexes form in vivo.

DISASSEMBLY OF SNARE CORE COMPLEXES

Because the core complex is extremely stable, it is not surprising that cells have evolved a specialized chaperone whose function is to dissociate the SNAREs in the core complex under ATP hydrolysis. This chaperone is

N-ethyl maleimide sensitive factor (NSF), which acts in conjunction with adaptor proteins termed soluble NSF attachment proteins (SNAPs) (no relation to SNAP-25). Both NSF and SNAPs are structurally and functionally conserved in evolution (their yeast counterparts are known as Sec 18 and Sec 17, respectively) (118). They were originally identified as soluble factors essential for the reconstitution of vesicular transport between Golgi compartments and are now known to function as SNARE complex chaperones in virtually all intracellular transport steps (119). NSF belongs to the AAA protein superfamily (ATPases associated with various cellular activities), which are characterized by a highly conserved ATP binding module (120). Although there are no obvious common denominators for the activities and targets of these diverse ATPases, it has been speculated that all AAA proteins use ATP hydrolysis to change the conformation of target proteins, i.e. that they function as molecular chaperones.

NSF is a hexamer (104, 121). The subunits contain three distinct domains: an N-terminal domain (N-domain) responsible for substrate binding and two nucleotide binding domains, referred to as D1 and D2 domain (122, 123). ATP hydrolysis by the D1 domain is crucial for NSF's catalytic activity, whereas the D2 domain is holding the hexamer together and contains little ATPase activity (124, 125). As revealed by the recently solved crystal structure, the D2 domain consists of a hexagonally shaped pinwheel with perfect symmetry (126, 127). In the middle, there is a hole that is surrounded by side groups bearing negative charges that may interact with the positively charged N-terminal part of the ternary complex. Furthermore, the r-phosphate of ATP interacts directly with a lysyl residue of the neighboring subunit, contributing to the stabilization of the oligomer that explains why NSF dissociates into monomers in the absence of nucleotides (127).

Electron microscopic imaging of NSF and NSF mutants in the ATP- and ADP-bound state revealed major conformational changes during the catalytic cycle. In the ADP form, the molecule appears as a symmetrical barrel that consists of two hexameric rings, the D2 and D1 domains, stacked on top of each other, whereas the N-domains fill in around these rings. In the ATP form, the N-domains form ball-like extensions appearing as "feet" surrounding the central rings (104).

Three variants of SNAPs exist in mammals, referred to as a-, b-, and r-SNAP, respectively (128, 129). In in vitro transport reactions a-SNAP and the brainspecific b-SNAP (which share >80[percent] homology) can substitute for each other (130, 131). Thus, they are probably functionally equivalent (132). A specific role of b-SNAP in synaptic vesicle exocytosis was postulated based on its in vitro association with synaptotagmin (133). r-SNAP enhances binding of a-SNAP to membranes but is inefficient in supporting in vitro transport reactions (129).

SNAPs need to bind to the SNARE complex before NSF can bind. Binding induces a conformational change in r-SNAP that can also be triggered by adsorption to plastic surfaces (134). SNAP binding stimulates the ATPase activity of NSF. Electron microscopic imaging has shown that r-SNAP binds laterally to the SNARE complex, whereas NSF binds to the end distal to the transmembrane domains (135).

Although SNAPs and NSF show a preference for assembled SNARE complexes (136), they also bind to syntaxin and (with lower affinity) SNAP-25 alone but not to synaptobrevin and other R-SNAREs (97, 112, 137). Like the ternary complex, these "partial" complexes fall apart upon ATP hydrolysis by NSF (137). Interestingly, NSF' s ATPase activity is stimulated more than 10-fold when acting upon syntaxin or SNAP-25 but only very moderately when acting upon the ternary complex (138). Perhaps more work is needed to unwind the tight helical bundle of the ternary complex resulting in a kinetic "slowdown" of the reaction.

Little is known about the conformations of the SNARE proteins after being acted upon by NSF and SNAPs. For syntaxin, a metastable state appears to be reached that prevents it from reassociating with the SNARE partners (137), possibly because of an interaction between the N-terminal and the homology domains (see above). The N-terminal domain is not required, however, for the disassembly reaction since the core complex is also disassembled by NSF and SNAPs (103). In yeast vacuolar fusion, a small heterodimeric protein, LMA1, is transferred from NSF to the syntaxin homologue Vam3p during ATP-cleavage that is thought to stabilize Vam3p in an active conformation (139). LMA1 is essential for reconstituting vacuolar

fusion in vitro. It is not known, however, whether other Q-SNAREs require similar cofactors.

As far as known, NSF operates on all SNARE complexes. However, the AAA family proteins p97/VCP (mammals) and Cdc48p (yeasts) are involved in specific fusion events where NSF cannot substitute, e.g. during postmitotic reassembly of the Golgi apparatus (140) and the reassembly of the nuclear envelope (141). Like NSF, p97 needs a cofactor known as p47 (142). Recently, p97 and Cdc48p have been demonstrated to bind to syntaxin 5 and Ufe1p, respectively, apparently involving complexes different from those acted upon by NSF in the same in vitro assay (143, 144). Although the effects of these interactions are not yet known, it has been speculated that p97/Cdc48 may break up Q-Q-SNARE complexes in certain homotypic fusion reactions that do not require R-SNAREs, whereas NSF would be specialized on the disassembly of Q-R-SNARE complexes (143).

TWO DISTINCT PROTEIN INTERACTION DOMAINS OF SYNTAXIN-LIKE SNARES

As mentioned above, syntaxins contain a characteristic N-terminal domain in addition to the SNARE motif. The N-terminal domain varies between syntaxin isoforms dependent on the localization of their respective trafficking steps. The N-terminal domain of syntaxin 1 resembles those of a subset of syntaxins thought to operate at the plasma membrane, such as syntaxins 2, 3, and 4 and yeast Sso1p and Sso2p (see 106 for sequence comparison). These similarities suggest that this domain of syntaxin has a conserved function specific for exocytosis. The three-dimensional structure of the N-terminal domain of syntaxin 1 has recently been solved by using NMR spectroscopy (106). The domain is composed of a twisted three-helical bundle, an unexpected finding because sequence analyses had only predicted two helices. The third helix, however, is highly conserved and binds to the two other helices to form a deep groove between the second and third helix. **Mapping** of the conserved sequences onto the three-dimensional structure showed that the groove is highly conserved, suggesting that it represents a binding site.

The presence of an evolutionarily conserved autonomously folding N-terminal domain in syntaxins indicates that these proteins have additional functions besides forming core complexes as SNAREs. Two different functions for this N-terminal domain have been proposed that may be related: First, studies on syntaxin 1 and the yeast syntaxin 1 homolog Sso1p showed that the N-terminal domain may fold back onto the SNARE motif if the SNARE motif is not assembled into a core complex and may interfere with the formation of the core complex (115, 145) (I. Dulubova, J. Rizo, et al, personal communication). Second, the N-terminal domain of syntaxin 1 binds to several proteins that are essential for synaptic vesicle exocytosis, namely munc 18-1, munc 13-1, and synaptotagmin 1 reviewed by Suhof (71) . Thus, it is likely that syntaxins participate in at least two distinct essential protein complexes that use either the N-terminal domain (binding a variety of proteins) or the SNARE motif (core complex formation). It should be noted that syntaxin can undergo associations with more than a dozen additional proteins, but a discussion of these interactions is beyond the scope of this chapter.

FUNCTION OF SNARES LOCALIZED TO MULTIPLE COMPARTMENTS

If SNAREs function in all cellular fusion reactions, they are expected to be present in the membranes of all intracellular compartments. Indeed, SNAREs are widely distributed in cells, and each SNARE exhibits a characteristic subcellular distribution (Table 1). SNAREs are often associated with transport vesicles and shuttle between trafficking compartments. As a result, many SNAREs are present on multiple intracellular compartments. Therefore, it is not sufficient to localize a SNARE in order to determine its site of action. For instance, SNAP-25 and syntaxin are distributed over the entire neuronal plasma membrane in adult animals, including myelinated axons (146). Nevertheless, in mature neurons, these SNAREs are only known to function at the synapse (see below), a location that contains a small minority of the total pool of these SNAREs.

SNARE proteins are sorted to their intracellular destinations by unknown mechanisms. Synaptobrevin (and probably also other SNAREs containing **C - terminal** transmembrane regions) is post-translationally

inserted into the membrane of the endoplasmic reticulum and then transported to the synapse (147). Insertion is ATP dependent and requires proteins different from the translocation complex needed for proteins with a genuine signal sequence. ER processing was also found for an invertebrate synaptobrevin variant that contains an extended luminal domain (148). Sorting of membrane-associated SNAREs probably involves cytoplasmic sequences and transmembrane regions for those SNAREs, as exemplified in the case of synaptobrevin and its close homolog cellubrevin (149-152). In synaptobrevin, the SNARE motif was implicated in sorting (149) However, mutations in the SNARE motifs disturb SNARE complex assembly or disassembly which may result in mislocalizations that are unrelated to protein sorting. Thus, it remains unclear if the sorting determinants that were identified in the SNARE motif of synaptobrevin (149) correspond to a genuine sorting sequence.

ESSENTIAL FOR FUSION

The first definitive clue to the function of SNARE complexes was obtained when the neuronal SNAREs synaptobrevin, syntaxin, and SNAP-25 were identified as targets for **botulinum** and tetanus neurotoxins (153-161). These toxins represent bacterial proteins that are composed of two polypetide **chains** . The **heavy chain** mediates the uptake of the toxins into the presynaptic nerve terminal where the light chain inhibits neurotransmitter release (for review, see e.g. 160-162). The toxins are highly potent in inhibiting vesicle exocytosis. However, poisoned nerve terminals exhibit no morphological changes and still contain abundant amounts of synaptic vesicles attached to the plasmalemma.

PA Tetanus toxin and the various types of **botulinum** neurotoxins are homologous to each other but proteolyze different substrates (synaptobrevin, SNAP-25, and syntaxin) or different sites in the same substrate (160, 161). Although fully assembled core complexes are resistant to toxin cleavage (96), all cleavage sites **map** to two narrow bands in the **C - terminal** region of the core complex structure (93). Apparently, the target structure but not the cleavage sites were preserved during evolution of the toxins. Regardless of the cleavage site, toxin-mediated SNARE cleavage prevents the assembly of a stable core complex and, in the case of synaptobrevin and syntaxin, detaches the SNARE from the membrane. Although SNAREs probably have other activities besides core complex assembly, the strong link between block of exocytosis and disruption of core complex formation suggests that the assembly of the core complex is a critical event in membrane fusion.

Mutations in yeast SNAREs and in vitro transport assays confirmed that SNARE complexes are essential for fusion but not for membrane attachment (78, 163, 164). In these systems, transport vesicles become "tethered" by the activity of Rab proteins (see below). Thereafter, SNARE core complexes assemble. As discussed above, mutations affecting core complex packing result in more or less severe loss-of-function phenotypes, supporting the key role of assembly in fusion.

Although it is now known that some SNAREs participate in more than one transport step, mutations of yeast SNAREs were instrumental in documenting functional specificity of SNAREs for a given step. When a SNARE shuttles between multiple compartments, e.g. during anterograde and retrograde transport, it may be operating in one of the associated fusion steps and excluded from others. For example, the neuronal SNAREs syntaxin, SNAP-25, and synaptobrevin are abundantly present on synaptic early endosomes but do not participate in the NSF-dependent fusion of these organelles (165, 166). Thus, the involvement of SNARE proteins in a given fusion step must be tightly regulated, and there must be mechanisms that select the SNAREs participating in a given trafficking step.

Where in the sequence of events leading to fusion do SNARE complexes assemble and is assembly sufficient to drive a complete fusion reaction? Evidence derived from both the study of synaptic transmission and of yeast vacuolar fusion suggests that SNARE complexes form early and by themselves cannot drive a fusion reaction to completion.

Synapses Synapses contain an exocytosis-competent ("readily releasable") vesicle pool that is membrane attached. Exocytosis of this pool can be induced by applying solutions of high osmolarity, e.g. hypertonic sucrose. Sucrose-mediated exocytosis is dependent on SNAREs, but membrane attachment is not (167, 168). Under physiological conditions,

calcium entry into the synapse triggers exocytosis of vesicles from the readily releasable pool with a delay of only 0.1-0.5 ms. Interestingly, each calcium signal releases only a small fraction of the readily releasable pool (169, 170). In other words, all vesicles undergo a SNARE-dependent maturation process after membrane attachment (prefusion). The SNARE-dependent prefusion makes the vesicles competent to respond to sucrose and calcium but is not sufficient to release neurotransmitters (i.e. open a fusion pore) without triggering. It is indeed difficult to interpret this data other than assuming that the SNAREs are assembled at this state.

The state at which synaptic vesicles are arrested after prefusion is unclear but may involve hemifusion. In adrenal chromaffin cells, toxins block exocytosis of all vesicle pools within minutes after injection, including the small subset of "release-ready" vesicles that need only milliseconds to fuse after the arrival of the Ca2+-signal (171). Reconstitution experiments with recombinant syntaxin, SNAP-25 (which, however, was not palmitoylated), and synaptobrevin showed that these proteins can mediate slow mixing of the outer leaflets of phospholipid vesicles (172). Thus, SNARE complex assembly may drive establishment of a hemifusion stalk. In the synapse, such a fusion intermediate may be stabilized by the unusually high concentrations of plasmalogen-phosphatidylethanolamine in the cytoplasmic leaflet of synaptic vesicles (K. Stenius, T. Glonek & R. Jahn, unpublished observations), a cone-shaped lipid that stabilizes stalks (173).

The question arises of what keeps prefused synaptic vesicles from progressing to fusion pore opening, or conversely, which mechanisms normally mediate this progression. The only protein that is currently known to be essential for this progression is synaptotagmin. Synaptotagmin is a Ca2+-binding membrane protein of synaptic vesicles that is required for fast Ca2+-activated exocytosis but not for sucrose-triggered exocytosis (174). Thus, synaptotagmin must be intimately connected with the final opening of the fusion pore. Intriguingly, synaptotagmin binds to syntaxin (175-177), to itself (178, 179), and to phospholipids (180, 181) in a Ca2+-dependent manner. These interactions could potentially execute a calcium-dependent role in fusion pore opening. It has been suggested that this role is that of an inhibitory "clamp," but this is unlikely because in the absence of synaptotagmin, fusion is not disinhibited, but its calcium-dependent progression is impaired (174, 182).

Vacuolar Fusion During in vitro fusion of yeast vacuolar precursors, SNARE core complexes assemble after membrane attachment but can be completely dissociated before fusion completion (78). Using stage-specific assays that can each be blocked by specific inhibitors, SNAREs were shown to form "trans"-complexes (bridging the two fusing membranes; see below) after vesicles are attached to each other via a reaction involving the Rab protein Ypt7p. These trans-complexes stably connect vesicles, but fusion can be arrested by phosphatase inhibitors and amphiphilic peptides such as mastoparan. Subsequent disassembly of the trans-SNARE complexes by excess amounts of NSF and SNAPs does not markedly inhibit fusion completion after release of the fusion block, arguing that core complexes are no longer needed for this step and that additional unknown proteins are involved. Surprisingly, calmodulin has recently been implicated in these late steps, suggesting that fusion completion requires a calmodulin-binding protein (183).

These data document that SNARE complex assembly alone is not sufficient for fusion completion in this system. However, it should be kept in mind that in the vacuole assay, fusion is measured by the transfer of a protease from the donor to the acceptor vacuole. In other words, the assay only reports fusion after the fusion pore is fully enlarged, whereas hemifusion intermediates including the transient opening of a fusion pore would not be detected. Thus, this data does not necessarily contradict the reconstitution studies of Weber et al (172), who measured outer leaflet mixing.

FUSION WITHOUT SNARE COMPLEXES

Although SNARE complexes are needed for all fusion reactions, fusion may proceed in the absence of SNAREs (particularly R-SNAREs), albeit at reduced rates and only under special circumstances. For instance, in Drosophila deletion of synaptobrevin resulted in a severe depression of evoked release, but spontaneous fusion events were still present (184, 185). Along

the same lines, deletion of synaptobrevin in Caenorhabditis elegans resulted in an animal that still retained some reduced ability to move (186), whereas syntaxin-null mutants are completely paralyzed (187). Furthermore, yeast cells lacking the synaptobrevin homologs Snc1p and Snc2p are still viable under certain conditions (188). These snc mutants are very sick but can be reverted to wild-type phenotypes by mutations in two separate but synergistic suppressor genes, elo2 and elo3. These genes code for enzymes involved in phospholipid biosynthesis and mediate the elongation of very-long-chain fatty acids and their subsequent incorporation into ceramide and inositol sphingolipids (188). Thus, the function of these R-SNAREs in fusion becomes expendable when the lipid composition of the membranes is changed.

Finally, in a cell-free assay, cortical granules of sea urchin undergo Ca2+-mediated fusion with the plasma membrane in the absence of cytosolic factors reviewed by Avery et al (189) . Fusion is mediated by SNARE proteins (190), but when the granules are centrifuged onto protein-free liposomes or plasma membranes that were treated with proteases before the experiment, fusion can still be induced by Ca2+. Thus, fusion proceeds in the absence of any protein in the target membrane (191) and fusion becomes independent of SNARE complex assembly and disassembly (192). Possibly, the application of mechanical force pressing the fusing membranes together bypasses SNARE action, thus revealing late-acting fusogens operating downstream of the SNAREs.

NANOMECHANICS OF SNARES: A MODEL FOR FUNCTION

Based on the results described above, a model for the function of SNARE core complex assembly can be proposed. This model postulates that SNARE complexes assemble after membrane attachment. Complexes assemble between membrane-anchored SNARE partners in the two membranes destined to fuse (trans-complex). During assembly the membranes are pulled very closely together, a prerequisite for initiating fusion (104, 193, 194). Assembly causes a stalk hemifusion intermediate, possibly associated with lipid mixing of the proximal membrane leaflets. Thereafter, the complex is expendable and can be fully dissociated without affecting the further progress of the fusion reaction. Fusion completion is performed by unknown downstream proteins that may not be conserved between different fusion events.

The proposed mechanism bears striking similarities with the mechanisms discussed for viral fusion proteins such as HA (see above). The essential step in both HA- and SNARE-mediated fusion would be the exertion of a pulling force that connects the membranes at locally confined spots. In both cases, the proteins need to be anchored in the two fusing membranes, and the conformational changes lead to a close juxtapositioning of the membrane anchors once the "relaxed" conformation is reached. Strikingly, the relaxed conformations are characterized by thermostable extended helical bundles of which the SNARE complex is the longest (50; see also above). Apparently, the conformational changes not only provide the energy for the pulling force but also produce stiff rods that may serve as levers or as anchor points during the exertion of force. Viral fusion proteins, however, are designed for "one shot only." Charging them requires an irreversible step (proteolysis) in contrast to the SNARE proteins that are recycled into an active state by NSF and g-SNAP at the expense of ATP. Interestingly, partial activation of HA leads to a stable hemifusion state, which in intracellular fusion may be a physiological intermediate.

SNARE HYPOTHESIS

The SNARE hypothesis (83, 119) was an early attempt to integrate the assembly-disassembly cycle of SNAREs into a coherent concept of membrane fusion. Although the hypothesis is presently primarily of historical interest, it is still frequently cited. The SNARE hypothesis postulated that the assembly of SNAREs mediates the attachment of membranes before fusion and that NSF-driven disassembly would lead to fusion completion. Furthermore, the specificity of v-SNARE/t-SNARE interactions was thought to determine the specificity of intracellular membrane traffic, i.e. that a single SNARE would function only in a single trafficking step (119). In retrospect, however, none of these predictions have been confirmed.

1. As discussed above, SNARE complexes function downstream of membrane attachment. For neuronal exocytosis, this was already known at the time the SNARE hypothesis was formulated reviewed by Sudhof et al (159) .

2. SNAREs form parallel, not antiparallel, complexes (see above). Furthermore, complex formation between SNAREs is rather promiscuous. Even SNAREs that probably do not interact with each other in vivo tightly bind to each other in vitro. For instance, synaptobrevin 2 binds to several syntaxins (98) and forms stable complexes with SNAP-23 (195). Furthermore, an only distantly related R-SNARE, endobrevin, is capable of forming a complex with the neuronal proteins SNAP-25 and syntaxin, which has properties very similar to those of the genuine complex with synaptobrevin (D. Fasshauer, W. Antonin, and R. Jahn, manuscript in preparation). Although the properties of these complexes and their in vivo relevance need to be further characterized, it is evident that the ability of SNARE proteins to form NSF-sensitive complexes is far less specific than postulated in the original SNARE hypothesis.

3. SNAREs can function in multiple trafficking steps. This is best documented in yeasts for the Q-SNAREs Vti1p and Sed5p, which are each involved in at least three distinct reactions reviewed by Gotte et al (196)!. Immunoprecipitation of SNARE complexes from yeasts revealed multiple complexes of Sed5p and Vti1p, supporting the notion that these proteins are involved in multiple trafficking steps (see 197 for a more detailed overview). Similar complexes were also found with their mammalian counterparts (198). Finally, SNAREs may substitute for each other that normally function in different trafficking steps. For instance, in vesicular transport from the Golgi apparatus to the vacuole, overexpression of Vam3p can partially compensate for a loss of Pep12p and vice versa (199, 200). Overexpression may overwhelm the intracellular sorting process, resulting in partial mislocalization, which may explain these phenotypes (201).

4. NSF does not participate in fusion itself. For instance, in Ca2+-triggered exocytosis of permeabilized neuroendocrine cells, ATP and NSF are not needed for the final steps in exocytosis but rather for fusion setup (202-204). Furthermore, a comparison of temperature-sensitive mutants of NSF (comatose) and syntaxin in Drosophila yielded evidence that NSF operates before syntaxin in exocytosis and that the defect in syntaxin was caused by its inability to form core complexes (205). Finally, in yeast vacuolar fusion Sec17p and Sec18p, the homologs of the mammalian a-SNAP and NSF are needed for an early stage of the reaction and become expendable before membrane attachment (163, 206). It should be noted that NSF's action on SNAREs is reversible and, at least in vitro, short-lived. Core complexes readily reassemble in the membrane plane of a single vesicle (111, 207, 208), rendering SNAREs inactive for fusion (nonconstructive "cis"-complexes). Continuous activity of NSF may be required to maintain SNAREs in a fusion-competent state. Furthermore, individual Q-SNAREs may relax into an inactive conformation without binding to other SNAREs, needing NSF's action to reaquire fusion competence.

SEC1/MUNC18 FAMILY OF PROTEINS

DEFINITION OF THE SM PROTEIN FAMILY

Members of the sec1/munc18 (SM) protein family were independently discovered genetically in yeasts (Sly1p, Sec1p) and Caenorhabditis elegans (unc18), and biochemically in vertebrates (munc18-1) (209-214). All SM proteins are hydrophilic proteins of 60-70 kDa that are devoid of recognizable subdomains. In pairwise comparisons, most SM proteins exhibit only 20-22[percent] sequence identity that is distributed rather evenly over the entire sequences of SM proteins. In yeasts, four SM proteins are known (Sec1p, Sly1p, Slp1p/Vps33p/Vam5p, and Vps45p/Stt10p) (214-219), which each participate in specific membrane trafficking steps: Sec1p acts exclusively at the plasma membrane, Sly1p is active in fusion events in the endoplasmic reticulum and Golgi apparatus, and Vps33p and Vps45p are involved in endosomal and vacuolar membrane traffic, respectively. In vertebrates, three SM proteins that act at the plasma membrane have been described (munc18-1, -2, and -3, also referred to as munc18a, 18b, and 18c) (211-213, 220, 221). In addition, there are at least two mammalian isoforms for Vps33p and one each for Sly1p and Vps45p (222-227). Probably, additional SM proteins remain to be discovered.

FUNCTION OF SM PROTEINS

Several results show that SM proteins perform an essential role in membrane fusion itself rather than being primarily involved in the regulation of fusion. In yeasts, mutations in the genes for various SM proteins result in a complete block in fusion. For example, in sec1 mutants, exocytosis is inhibited, and sly1 mutants exhibit a complete block in the export of proteins from the ER (228, 229). In mice, deletion of munc18-1 is lethal because it prevents vesicle exocytosis (M Verhage & TC Sudhof, unpublished observations). However, membrane attachment of vesicles is normal. Similar results were obtained in Drosophila mutants affecting the munc18 homolog Rop (230). These results suggest that SM proteins act in prefusion after membrane attachment but probably before formation of a fusion pore.

What is the function of SM proteins in fusion? In view of their essential nature, it is surprising that so little is known about the features of these proteins. Their sequences reveal no motif that would predict a function. For one family member, Vps33p, residues resembling an ATP-binding site were detected. Although Vps33p was shown to bind ATP in biochemical studies (231), the putative ATP-binding site is not conserved in other SM proteins, and munc18-1 does not bind ATP in direct-binding studies (TC Sudhof, unpublished data). The only known common property of SM proteins is that they interact with Q-SNAREs of the syntaxin family. Recent studies have examined the interaction of syntaxin 1 with munc18-1 in detail. Munc18-1 binds to the N-terminal domain of syntaxin 1 and to the N-terminal part of the adjacent SNARE motif. Syntaxin 1 does not simultaneously bind to munc18-1 and to SNAP-25 (232) presumably because their binding sites overlap in the SNARE motif. As a result, syntaxin 1 participates in two alternative protein complexes that both may be essential for fusion: the munc18-1/syntaxin 1 complex and the SNARE core complex. Probably other syntaxins undergo similar mutually exclusive interactions with SM proteins and SNAREs but these have not yet been studied.

It thus seems likely that SM proteins operate in fusion by binding to syntaxin-like Q-SNAREs, although other modes of action cannot be excluded (see below). Furthermore, the structural consequences of the interactions are unclear. It is unknown at present if the binding of SM proteins to syntaxins precedes or follows SNARE assembly in fusion or whether it is involved in stabilizing intermediate stages of SNARE assembly/disassembly.

PROTEINS THAT MAY FUNCTIONALLY INTERACT WITH SM PROTEINS

In vacuolar membrane fusion and at the synapse, SM proteins undergo protein-protein interactions that could be important for fusion. Confusingly, however, the binding partners of different SM proteins, as far as characterized, have little in common. Either some of these interactions are not central to SM protein function or the molecular mechanisms mediating their function differ between various SM proteins.

In vacuolar membrane traffic in yeasts, the two SM proteins Vps33 and Vps45 genetically interact with proteins containing zinc finger sequences of the RING type (233-235). RING-type zinc fingers are identified by highly conserved basic sequences surrounding the central zinc-coordinating cysteine residues. The two RING finger proteins Vps11p and Vps18p bind to each other and to a third protein, Vps16p, which in turn appears to bind to Vps33 (235). Vps33, conversely, seems to interact with the syntaxinlike Q-SNARE Vam3 in vacuole fusion. Although the complexes between these proteins appear to be transient, mutations in all of these genes result in similar phenotypes with a block in vacuole fusion, suggesting that their interactions are functionally important. In addition, the syntaxinlike Q-SNARE Pep12p forms a complex with Vps45p and with another RING finger protein, Vac1p/Vps8p (234, 236). However, here it is unclear if Vac1p binds directly or indirectly to the SM protein or to the SNARE. Interestingly, the localization of Vac1p on the endosomal membrane depends on a Rab protein called Vps21p/Ypt51p, supporting a functional connection between SM proteins and Rab proteins (237). Such a functional connection is supported by the observation that sly1 mutants are multicopy suppressors of Ypt1p mutants in the ER fusion reaction (214).

The results obtained with Vps33p and Vps45p suggested a general interaction of SM proteins with RING finger proteins. It was thus disappointing that in yeasts, no essential RING finger protein was

discovered outside of the endosomal/vacuolar system. In yeasts, the only protein known to directly bind to Sec1p is a small protein called Mso1p that is not homologous to any other known protein (238). Studies of the binding partners of the synaptic SM protein munc18-1 yielded a set of proteins that was completely different from that observed for the yeast vacuolar system. Two protein families were identified. One is a family of multidomain proteins called mints (239, 240). Mints contain an N-terminal munc18-1 binding domain that in mint 1 is followed by a sequence that binds to CASK, a cellular junction protein enriched in synapses (241). This result suggested that mint 1 could function to couple exocytosis to synaptic cell adhesion but helped little in the identification of the mechanism by which munc18-1 acts in membrane fusion. The second protein family identified was a doublet of C2 domain proteins called Doc2A and Doc2B (242). Doc2s contain two C2 domains that may bind calcium. Their first C2 domain specifically binds munc18-1. However, the localization of Doc2s is unknown, and their relation to membrane traffic, if any, is uncertain.

RAB PROTEINS IN MEMBRANE FUSION

RAB PROTEINS CYCLE ON AND OFF MEMBRANES

Rab proteins are low-molecular-weight GTP-binding proteins that are attached to target membranes via two **C - terminal** geranylgeranyl groups. One of the best-studied Rab proteins is Rab3A, which is abundantly present in presynaptic nerve terminals. In the GTP-bound form, Rab3A is associated with synaptic vesicles. Some time during or after exocytosis, GTP on Rab3A is hydrolyzed to GDP, and Rab3A dissociates from the vesicles, probably by binding to a protein called GDI. Thereafter, Rab3A rebinds to the vesicles that have reendocytosed under GDP-to-GTP exchange reviewed in Sudhof (243) . It is likely that other Rab proteins undergo a similar cycle of membrane association and dissociation coupled to GTP binding and hydrolysis, as evidenced by the essential nature of GDI in yeasts (244). This association-dissociation cycle is probably the reason that Rab proteins are attached to membranes by a geranylgeranyl modification instead of a transmembrane region. However, the biological necessity for this association-dissociation cycle is unclear.

FUNCTION OF RAB PROTEINS IN FUSION

The yeast genome contains ten rab genes and one additional rab-like gene reviewed by Lazar et al (245) . More than 40 Rab proteins have been described in mammals (246, 247). Various Rab proteins are associated with specific subcellular organelles, making them useful markers for these compartments. Extensive evidence in yeasts and mammals suggests that Rab proteins are involved in membrane fusion. In yeasts, a Rab protein encoded by the sec4 gene is required for exocytosis, and other Rab proteins encoded by the ypt1, vps21/ypt51, and ypt7 genes are necessary for normal trafficking from the endoplasmic reticulum to the Golgi apparatus, from the Golgi apparatus to the endosome, and from the Golgi apparatus or endosome to the vacuole, respectively (248-252). In the absence of these Rab proteins, fusion is blocked at defined stages. Although no evidence for an essential role of Rab proteins in a mammalian fusion reaction has been presented, several dominant negative mutants of Rab proteins block defined fusion reactions (see e.g. 253-257). This agrees with the conclusions from yeast studies that Rab proteins function in fusion.

At which stage of the fusion reaction do Rab proteins act, and what do they do? Two systems were used for particularly detailed analyses of Rab protein function: In mice, the effects of deleting Rab3A were studied (258-261), and in yeasts the role of Sec4, Ypt1p, and Ypt7p in exocytosis, ER to Golgi fusion, and vacuole fusion were explored (78, 164, 248). The two systems gave dramatically different results that at present are difficult to reconcile for a unitary action of Rab proteins.

Yeasts In vitro assays revealed that Ypt1p is essential for attaching ER-derived vesicles to Golgi membranes, and Ypt7p for tethering vacuolar membranes. In each case, membrane attachment was shown to occur before SNARE complex assembly and to be independent of SNAREs (78, 164). Thus, the various Rab proteins in yeast are probably essential for fusion reactions because they mediate membrane recognition and attachment. However, although the in vitro assays provide convincing results, several puzzling issues

need to be resolved. First, in the in vitro assays, one mechanism to inactivate Rab proteins is to remove them with the protein GDI. GDI recognizes only GDP-bound Rab proteins and allows their dissociation from membranes (262). This raises the question of whether Rab proteins are present in these assays as GDP- or GTP-bound proteins. Demonstration that only GTP-bound Rab proteins function in the membrane attachment assays, and identification of their targets would be very useful. Second, although membrane attachment in the in vitro assays is completely independent of SNAREs, curiously, it requires SNARE complex disassembly before Rab protein mediated membrane attachment. Furthermore, there are genetic interactions between the rab genes and SNAREs. For example, Sec4p mutants can be suppressed by overexpression of Sec9p, the SNAP-25 homolog, and Ypt1p by a Sly1p mutant (214, 263). Because the phenotype caused by the loss of a Rab protein in yeasts can be suppressed by point mutations in other genes, the function of Rab proteins is probably not essential for fusion as such.

Synapses Synaptic vesicles contain three isoforms of Rab3 as major components. Rab3A is present in all synapses, and Rab3B and Rab3C are present in subsets of synapses (264, 265; R. Jahn and T. C. Sudhof, unpublished observations). In addition, the vesicles contain Rab5, which is known to function in endosomes (266, 267). In view of the essential nature of rab genes in yeasts, it was surprising that synaptic vesicle exocytosis was only moderately impaired in mice lacking Rab3A (258). Equally surprising, fusion triggered by hypertonic sucrose, which is thought to reflect vesicles that are attached and prefused via SNARE complexes, was unchanged in the Rab3A-deficient synapses (259). The rate at which the sucrose-releasable vesicle pool is replenished is thought to reflect membrane attachment and prefusion (which involves SNAREs). This rate was also normal in the Rab3A-deficient synapses, suggesting that Rab3A is not involved in trafficking before SNARE complex assembly. However, these synapses did exhibit a phenotype: Calcium-triggered fusion was enhanced, and mossy fiber long-term potentiation, a long-lasting form of presynaptic plasticity, was abolished (259-261). These data showed that at the synapse, the function of Rab3A becomes manifest after core complex assembly from SNAREs. Is it possible that Rab3A is redundant with other forms of Rab3? This appears unlikely because in some of the synapses analyzed in the Rab3A knockout mice, no other isoforms appear to be present (260). Furthermore, mutations in the single rab3 gene encoded in the Caenorhabditis elegans genome cause a similarly mild phenotype as rab3A deletions in mice (268). Thus, Rab3 seems to have similarly restricted functions in regulating fusion in mice and worms, with the electrophysiological characteristics of the phenotype indicating a role distal of SNARE complex assembly.

At present there is no ready explanation for the differences in apparent Rab function between in vitro assays in yeasts and synaptic electrophysiology. However, it is still possible that Rabs in these two systems and in all other trafficking steps perform similar roles. The fact that Rabs are essential in several yeast trafficking steps but not at the synapse is most easily explained; after all, even the function of essential rabs in yeasts can be suppressed by point mutations in, or overexpression of, other genes. Thus, it is easy to imagine that a point mutation in an interacting gene transformed the role of Rab3A from an essential function to a modulatory function e.g. in Sly1p (214) . More difficult is the apparent difference in the point of action. The major problem here is that both assay systems are not definitive. As described above, if Rab proteins in yeasts were essential for membrane attachment, it is difficult to explain why SNARE disassembly is essential for their function and why point mutations in SNAREs or related proteins suppress their loss of function. Conversely, the fact that the functional effects of Rab3A deficiency become apparent at the synapse during the calcium-triggering stage does not prove that Rab proteins actually act here; their physical activity could be during an earlier stage that is only phenotypically apparent later. In the absence of a molecular understanding of the function of Rab proteins, it will be difficult to clarify their precise point of action.

MECHANISM OF ACTION OF RAB PROTEINS

As GTP-binding proteins, Rab proteins presumably function via GTP-dependent interactions with effector proteins. Putative Rab effectors are identified as proteins that bind only to a specific Rab protein and only bind in the presence of GTP. However, GTP-dependent binding proteins could also have other noneffector functions, and testing effector functions has proved

difficult. Search for GTP-dependent putative effectors for Rab proteins has led to a number of candidates. It is striking that there is no common theme in these candidate effectors with the exception of the putative Rab3 effectors and effectors for Rab5/Ypt51p (see below). Thus several of the candidate effectors may eventually turn out to have other functions.

Three distinct Rab3 effectors, called Rabphilin, NOC2, and RIM, were identified (269-271). These proteins share a common Rab-binding domain characterized by a zinc finger motif. In yeast endosome fusion, a putative effector protein was identified for Ypt51p/Vps21p in Vac1p (237). Two putative effectors were described for the corresponding mammalian protein, Rab5. These two potential effectors are EEA1 and rabaptin (272, 273); they share no sequence homology with each other, but EEA1 is very similar to vac1. EEA1 and vac1 contain zinc finger motifs homologous to the Rab3-binding zinc finger motif in rabphilin, RIM, and NOC, but the zinc finger in EEA1 and Vac1p appears to be involved in binding to lipids and not to Rab5 (233, 273, 274). As regards rabaptin, recent biochemical studies suggest that it may be involved in regulating GTP binding to Rab5, with its effect on in vitro fusion being an epiphenomenon (275). Potential Rab effectors have been described for other Rab proteins, but there is no biological correlation with activity, making it difficult to evaluate their validity.

As discussed above, the functions of Rab proteins intersect with those of SNAREs. However, there is little evidence for a direct interaction of a Rab protein with a SNARE or SM protein. The single report of a direct binding of a Rab protein, Ypt1p, to a SNARE, Sed5p, suggested that it competes with the SM protein Sly1p, which disagrees with the proposed binding constants (276). Thus, it seems likely that Rab proteins do not directly interact with SNAREs or SM proteins. Although the functions and activities of Rab proteins are unclear, it seems likely that they are universal regulators of the other two universal components of fusion reactions, SNAREs and SM proteins. It is too early to tell at this point if different Rab proteins will have distinct effectors and mechanisms of action. Although the current literature with several putative interactors involved in a wide variety of processes would suggest this, the data do not rule out the possibility that some of these putative interactors are artifacts and that a common theme will be found in the interactors for Rab proteins. Early indications for such a common theme are present in the finding that all genetic analyses of Rab proteins point to a common function, which intersects SNARE proteins, and that several Rab proteins specifically bind to a zinc finger motif.

PERSPECTIVE

Membrane fusion is one of the most fundamental processes in eukaryotic cells. In all cells, multiple fusion reactions occur at any given time, mediating such diverse functions as secretion, endocytosis, intracellular digestion in lysosomes, cell division, and adjustment of mitochondrial numbers to a cell's energy requirements. The mechanisms that govern cellular membrane fusion are now beginning to be understood. Work in recent years has identified a conserved framework of molecules that appear to be involved in all intracellular fusion reactions. These molecules are the SNAREs, SM (sec1/munc18 homologous) proteins, and Rab proteins. If it is confirmed that these proteins are obligatory components of all intracellular fusion reactions, then all intracellular fusion events will follow the same general mechanism. This mechanism needs to be highly adaptable when considering the vast diversity of intracellular fusion reactions.

However, we are only at the beginning of the task of elucidating fusion mechanisms. The most pressing problem in membrane fusion now is to understand what the obligatory components actually do. As discussed above, intracellular membrane fusion reactions can be divided into five phases: (a) Fusion set up involving activation of SNAREs and targeting of GTP-Rab proteins to the membrane. This phase may include other ATP-requiring steps. (b) Membrane attachment involving recognition of the membranes and their initial interactions. It is unclear if there is a unitary mechanism for this step since in yeasts, Rab proteins appear to function here, whereas at the synapse, the major Rab protein has no role in membrane attachment. Possibly, the major differences between various intracellular fusion

reactions lie in this step because this step must confer specificity to intracellular membrane traffic. (c) Prefusion involving generation of a hemifusion stalk under SNARE complex assembly. This step does not yet generate a fusion pore. (d) Completion of the fusion reaction with formation of a fusion pore, again by an unknown mechanism. It is unlikely that SM proteins function here because NSF-mediated disassembly of the SNARE complex is not essential for fusion. However, at the synapse the calcium-binding protein synaptotagmin is essential for triggering this step physiologically, suggesting that it may participate. (e) Dilation of the fusion pore to a fully expanded fusion hole with lipid mixing in both bilayers. Nothing is known about the molecular basis of this step. The stages of this model have corresponding stages in the HA-mediated fusion reaction. Here, fusion setup corresponds to the activation of HA by proteolytic cleavage yielding HA1 and HA2, attachment to the binding of HA to sialic acid on the target membrane, prefusion to the reaction performed by incompletely activated HA or HA lacking a transmembrane region, and the last two phases to the generation and expansion of the fusion pore. The comparison with the HA system shows that in spite of a complex series of steps, the reaction is principally simple. It reveals that a fusion peptide alone is not sufficient for pore formation and that additional mechanisms transform a prefusion state to a fusion pore. However, the model identifies only a single universal step in the fusion reaction performed by homologous proteins: the SNARE-mediated prefusion reaction. At present, the function of the SM and Rab proteins is uncertain, and the nanomechanics of fusion are unknown. Addressing these important issues is the major challenge for future studies.

Added material

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TABLE 1 SNARE proteins(FNa)

FOOTNOTES

a Abbreviations used are ER, endoplasmic reticulum; TMR, transmembrane region.

b Unless indicated otherwise, refers to sequences from Saccharomyces cerevisiae (yeast), rat or mouse (mammals), or Arabidopsis thaliana (plants). Data-bank searches indicated the presence of additional open reading frames containing SNARE sequences, particularly in plants. With few exceptions, we list only those for which expression has been demonstrated at the protein level.

Figure 1 Intermediates in membrane fusion: (a) membranes separated; (b) fusion stalk (void spaces in gray); (c) transbilayer contact; (d) opening of the fusion pore; (e) hemifusion diaphragm; (f) pore formation in the diaphragm. See text for details.

Figure 2 Model describing how the viral fusion proteins HA2 and gp41 fuse membranes. Only one subunit of each trimer is drawn. The extended intermediates are hypothetical. (Top row) HA2. A, B, C, D refer to a-helices. (Bottom row) gp41. N and C refer to the N- and **C - terminally** located a-helices. The fusion peptides are depicted as black circles. See text for details.

Figure 3 Structures of the N-terminal domains of syntaxin 1 and of the synaptic core complex (modified after References 92, 106). For comparison, the linear arrangement of the domains is shown for each protein in like colors.

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TEXT:

Since diphtheria toxin was isolated by Roux and Yersin in 1888 (1), microbial toxins have been recognized as the primary virulence factor(s) for a variety of pathogenic bacteria. Bacterial toxins have been defined as "soluble substances that alter the normal metabolism of host cells with deleterious effects on the host" (2). Indeed, the major symptoms associated with disease caused by Corynebacterium diphtheriae (diphtheria), Bordetella pertussis (whooping cough), Vibrio cholerae (cholera), Bacillus anthracis (anthrax), Clostridium **botulinum** (botulism), Clostridium tetani (tetanus), and enterohemorrhagic Escherichia coli (bloody diarrhea and hemolytic uremic syndrome) are all related to the activities of the toxins produced by these organisms. With the recognition of the central role of toxin in these and other diseases has come the application of inactive toxins (toxoids) as vaccines. Such toxoid vaccines have had an important positive impact on public health.

In this review, we provide a summary overview (Table) of a variety of bacterial toxins categorized according to mode of action: damaging cell membranes, inhibiting protein synthesis, activating second messenger pathways, inhibiting the release of neurotransmitters, or activating the host immune response. We also describe in detail seven toxins: Staphylococcus aureus a-toxin, Shiga toxin (Stx), cytotoxic necrotizing factor type 1 (CNF1), E. coli heatstable toxin (ST), **botulinum** and tetanus neurotoxins, and toxic-shock syndrome toxin (TSST) produced by S. aureus. We emphasize these toxins because they are produced by emerging (Stx of enterohemorrhagic E. coli) or reemerging (a-toxin of multidrug-resistant S. aureus) pathogens or illustrate different structures or modes of action (ST, CNF1, neurotoxins, and TSST).

WHEN IT RAINS, IT PORES

Many bacterial exotoxins have the capacity to damage the extracellular matrix or the plasma membrane of eukaryotic cells. The damage not only may result in the direct lysis of cells but also can facilitate bacterial spread through tissues. Toxins that mediate this cellular damage do so by either enzymatic hydrolysis or pore formation. Bacterial hyaluronidases, collagenases, and phospholipases have the capacity to degrade cellular membranes or matrices. Specific examples of these types of toxins include the a-toxin of Clostridium perfringens, which has phospholipase C activity; Streptococcus pyogenes streptokinase, which can hydrolyze plasminogen to plasmin and dissolve clots; and the clostridial collagenases (3-5). Pore-forming toxins, as the name suggests, disrupt the selective influx and efflux of ions across the plasma membrane by inserting a transmembrane pore. This group of toxins includes the RTX (repeats in toxin) toxins from gram-negative bacteria, streptolysin O produced by S. pyogenes, and the S. aureus a-toxin (described below).

S. aureus a-toxin can be considered the prototype of oligomerizing pore-forming cytotoxins. The a-toxin gene resides as a single copy on the

chromosome of most pathogenic S. aureus strains, and its expression is environmentally regulated at the transcriptional level by the staphylococcal accessory gene regulator (agr) locus (6,7). The a-toxin is synthesized as a 319 amino acid precursor molecule that contains an N-terminal signal sequence of 26 amino acids. The secreted mature toxin, or protomer, is a hydrophilic molecule that lacks cysteine residues and has a molecular mass of approximately 33 kDa (6-8). Recently, the crystallographic structure of the fully assembled a-toxin pore was solved (9). On the plasma membrane, seven toxin protomers assemble to form a 232-kDa mushroom-shaped heptamer comprising three distinct domains (Figure 1A) (9,10). The cap and rim domains of the a-toxin heptamer are situated at the surface of the plasma membrane, while the stem domain serves as the transmembrane channel.

Alpha-toxin is cytolytic to a variety of cell types, including human monocytes, lymphocytes, erythrocytes, platelets, and endothelial cells (6,8). For a-toxin to damage cellular membranes, three sequential events are required. Toxin protomers must first bind to target membranes by either unidentified high-affinity receptors or through nonspecific absorption to substances such as phosphotidylcholine or cholesterol on the lipid bilayer (6-8). Second, membrane-bound protomers must oligomerize into a nonlytic prepore heptamer complex. Third, the heptamer must undergo a series of conformational changes that create the stem domain of the toxin, which is then inserted into the membrane (9,10). The a-toxin pore allows the influx and efflux of small molecules and ions that eventually lead to the swelling and death of nucleated cells and the osmotic lysis of erythrocytes. Pore formation has also been shown to trigger secondary events that could promote development of pathologic sequelae. These events include endonuclease activation, increased platelet exocytosis, release of cytokines and inflammatory mediators, and production of eicosanoids (6,8). Several animal models have demonstrated that a-toxin is required for S. aureus virulence in these systems (6,8); however, the precise role of a-toxin in staphylococcal diseases in humans remains unclear.

STOP, IN THE NAME OF TOXIN

A second class of toxins intoxicates target cells by inhibiting protein synthesis. Substrates for toxins in this group are elongation factors and ribosomal RNA. Diphtheria toxin and Pseudomonas exotoxin A act by ADP-ribosylating elongation factor 2 (EF2) (11,12). The modified EF2 is no longer able to function in protein synthesis. Stxs, also called verotoxins, are produced by Shigella dysenteriae serotype 1 and the emerging pathogens designated Stx-producing E. coli (STEC). Stxs inactivate ribosomal RNA (by a mechanism described below) so that the affected ribosome can no longer interact with elongation factors (13,14). The inhibition of protein synthesis by this group of toxins ultimately results in death of the target cell.

Stxs are potent cytotoxins that can be divided into two **antigenically** distinct groups that share 50[percent] to 60[percent] homology: Stx/Stx1 and Stx2 (15-17). Stx and Stx1 are elaborated by S. dysenteriae serotype 1 and E. coli, respectively, and differ at only one amino acid. Stx2-type toxins have been found only in E. coli isolates and are quite diverse. While Stx2 is considered the prototype of this group, variants have been found that differ **antigenically** , in receptor specificity and in activation by intestinal mucus. Some of these attributes are the result of only one or two nucleotide differences in the toxin genes.

The stx of S. dysenteriae is invariably chromosomally located. The genes that encode Stx1 and Stx2 are carried chromosomally or by lysogenic bacteriophages. The genes that code for the A and B subunits of Stxs, stxA and stxB, are organized within an operon. The operator region of Stx/Stx1 (but not Stx2) contains a consensus fur box that is responsible for the iron-regulation of Stx and Stx1 production. Neither iron nor any other environmental factors examined affect the expression of Stx2. However, intestinal mucus enhances the activity of some Stx2 variants (18). The Stxs, which carry typical N-terminal leader sequences, are not actively secreted from the bacterial cell and are thought to be released into the milieu during cell lysis.

Stxs display an AB-toxin structure; an enzymatically active A subunit is noncovalently associated with a binding, or B, component. The crystal structures of the Stx1 B pentamer (19) and the Stx holotoxin have been

solved (20) (Figure 2). Other toxins that share this AB structure are the E. coli heat-labile toxin (21), cholera toxin, and pertussis toxin (22) (Figure 2). The molecular masses of mature Stx A and B monomeric subunits are approximately 35 kDa and 7.5 kDa, respectively, although holotoxin contains five B subunit molecules. The B subunit pentamer directs the binding of the holotoxin to sensitive eukaryotic cells via specific glycolipid receptors. Once internalized, the A polypeptide is cleaved into an enzymatically active A1 portion and an A2 portion; these fragments remain associated through a disulfide bond. The A2 portion serves to link the A1 fragment and the B pentamer.

The enzymatic A subunit acts as a specific N-glycosidase to cleave a single adenine residue from 28S ribosomal RNA (13,14). This depurination ultimately results in the inhibition of protein synthesis within intoxicated cells (Figure 1B). Prokaryotic ribosomes are as sensitive to the N-glycosidase activity of Stx as eukaryotic ribosomes (23).

STEC are considered emerging pathogens (24) because they were first described less than 20 years ago, during a 1983 outbreak of hemorrhagic colitis associated with undercooked hamburger (25,26). STEC O157:H7 causes approximately 20,000 cases of hemorrhagic colitis each year in the United States (27). Approximately 1,000 cases of the life-threatening sequelae hemolytic uremic syndrome and approximately 100 deaths are also attributed to E. coli O157:H7 annually in the United States (27).

DON'T SHOOT THE MESSENGER

Bacterial toxins can also target and alter the function of a variety of cellular proteins without directly killing the intoxicated cell. Toxin activation or modification of secondary messengers can cause dramatic alterations to signal transduction pathways critical in maintaining a variety of cellular functions. To demonstrate the diversity among the toxins that belong to this category, we will describe CNF type 1 and the heat-stable enterotoxins.

CYTOTOXIC NECROTIZING FACTOR (CNF)

CNF types 1 and 2 (CNF1/2) from E. coli belong to a group of bacterial toxins that modify Rho, a subfamily of small GTP-binding proteins that are regulators of the actin cytoskeleton (28,29). Most members of this toxin family, which includes the large clostridial cytotoxins and the C3 exoenzyme of C. **botulinum** , inactivate Rho (29). CNF1, CNF2, and the dermonecrotic toxins from Bordetella species form a unique subset in this family, since these toxins have the capacity to activate Rho (Figure 1C) (29-32). CNF1 and CNF2 share 99[percent] amino acid similarity; however, we will discuss only CNF1 in detail because of its association with extraintestinal E. coli infections in humans, most notably urinary tract infections.

The gene for CNF1 is chromosomally encoded and resides on a pathogenicity island in uropathogenic E. coli (33,34). The toxin is synthesized as a hydrophilic polypeptide of approximately 115 kDa that remains primarily cytoplasmic because of the lack of a signal sequence (33). Recent structure and function analysis of CNF1 indicates that the toxin has distinct binding and enyzmatic domains (35). The N-terminal half of CNF1, which includes two potential transmembrane domains, contains the cellular binding domain. This region of the molecule shows amino acid similarity to the Pasteurella multicoda toxin, a potent mitogen thought to be the etiologic agent of progressive atrophic rhinitis in pigs (33,35). The **C - terminal** portion of CNF1 represents the toxin's enzymatic domain and shows homology with dermonecrotic toxins in a 100-amino acid stretch that may represent the active site of the toxin (33,35).

Eukaryotic cells intoxicated with CNF1 exhibit membrane ruffling; the formation of focal adhesions and actin stress fibers; and DNA replication in the absence of cell division, a phenomenon that results in enlarged multinucleated cells (Figure 3). The drastic changes apparent in CNF1-treated cells are a result of the toxin's capacity to modify Rho (29,30,32). This modification has recently been identified as a deamidation of the glutamine residue at position 63 of Rho to a glutamic acid. This amino acid change produces a dominant active Rho protein unable to hydrolyze bound GTP (30,32). In vivo, CNF1 causes necrosis in rabbit skin following intradermal injection and persistent inflammation in a mouse

footpad assay (36). Epidemiologic data support the role of CNF1 as a virulence factor in human extraintestinal infections, although direct proof of the toxin's role in disease remains to be determined (29,37).

HEAT-STABLE TOXIN (ST)

Two families of diarrheagenic STs have been described: STa (or STI) and STb (or STII). Distinct STas are produced by a variety of enteric pathogenic organisms: enterotoxinogenic E. coli (ETEC) (the focus of this section), V. cholerae, Vibrio mimicus, Yersinia enterocolitica, Citrobacter freundii, and Klebsiella.

Strains of ETEC associated with human disease may produce either STa, heat-labile toxin I, or both. STas from ETEC isolates are related but distinct toxins (38). STh is produced by strains of human origin, while STp is found predominantly in porcine strains. The STa genes (estA) of ETEC are encoded within a transposable element and have been found on a variety of replicons (39,40). STa is translated as a precursor molecule of 72 amino acids and undergoes two cleavage events before the secretion of the mature form into the culture supernatant. Mature STs are small peptides that range from 17 to 53 amino acids. STh and STp contain 19 and 18 residues, respectively. STas share a conserved **C - terminal** region of 13 amino acids essential for toxicity and the heat-stable nature of the toxin. Six cysteine residues are present within this domain, and the three disulphide bonds formed between the cysteine residues are necessary for toxicity of the molecule. Binding of STa to its cellular receptor results in the stimulation of membrane-bound guanylate cyclase, which in turn leads to an increase in intracellular cyclic GMP (Figure 1C) (41). This increase in cyclic GMP affects electrolyte flux in the bowel; sodium absorption is inhibited and chloride secretion is stimulated. These ion flux changes result in the secretory diarrhea characteristic of ETEC infection. ETEC cause traveler's diarrhea and are a major source of childhood diarrhea in many parts of the world.

THE NERVE OF SOME TOXINS

The C. **botulinum** neurotoxins (BoNTs, serotypes A-G) and the C. tetani tetanus neurotoxin (TeNT) constitute another category of bacterial toxins on the basis of similarities in structure, enzymatic activity, and the targeting to cells of the nervous system. BoNTs are most commonly associated with infant and foodborne botulism and exist in nature as large complexes comprised of the neurotoxin and one or more associated proteins believed to provide protection and stability to the toxin molecule while in the gut (42,43). TeNT, which is synthesized from vegetative C. tetani in wounds, does not appear to form complexes with any other protein components (42,43).

The BoNTs and TeNT are either plasmid encoded (TeNT, BoNTs/A, G, and possibly B) or bacteriophage encoded (BoNTs/C, D, E, F), and the neurotoxins are synthesized as inactive polypeptides of 150 kDa (44). BoNTs and TeNT are released from lysed bacterial cells and then activated by the proteolytic cleavage of an exposed loop in the neurotoxin polypeptide (45). Each active neurotoxin molecule consists of a heavy (100 kDa) and light chain (50 kDa) linked by a single interchain disulphide bond (42,45). The **heavy chains** of both the BoNTs and TeNT contain two domains: a region necessary for toxin translocation located in the N-terminal half of the molecule, and a cell-binding domain located within the **C - terminus** of the **heavy chain** (45,46). The light chains of both the BoNTs and TeNT contain zinc-binding motifs required for the zinc-dependent protease activities of the molecules (45,46).

The cellular targets of the BoNTs and TeNT are a group of proteins required for docking and fusion of synaptic vesicles to presynaptic plasma membranes and therefore essential for the release of neurotransmitters. The BoNTs bind to receptors on the presynaptic membrane of motor neurons associated with the peripheral nervous system. Proteolysis of target proteins in these neurons inhibits the release of acetylcholine, thereby preventing muscle contraction (47,48). BoNTs/B, D, F, and G cleave the vesicle-associated membrane protein and synaptobrevin, BoNT/A and E target the synaptosomal-associated protein SNAP-25, and BoNT/C hydrolyzes syntaxin and SNAP-25 (42,45,46). TeNT affects the central nervous system and does so by entering two types of neurons. TeNT initially binds to receptors on the

presynaptic membrane of motor neurons but then migrates by retrograde vesicular transport to the spinal cord, where the neurotoxin can enter inhibitory interneurons (45,47). Cleavage of the vesicle-associated membrane protein and synaptobrevin in these neurons disrupts the release of glycine and gamma-amino-butyric acid, which, in turn, induces muscle contraction (47,48). The contrasting clinical manifestations of BoNT or TeNT intoxication (flaccid and spastic paralysis, respectively) are the direct result of the specific neurons affected and the type of neurotransmitters blocked (45-47).

BACTERIAL SUPERANTIGENS: TOO MUCH OF A GOOD THING

Several bacterial toxins can act directly on the T cells and **antigen** -presenting cells of the immune system. Impairment of the immunologic functions of these cells by toxin can lead to human disease. One large family of toxins in this category are the pyrogenic toxin superantigens (PTSAgs), whose hallmark biological activities include potent stimulation of the immune cell system, pyrogenicity, and enhancement of endotoxin shock (49-51). These stable, secreted toxins of 22 kDa to 30 kDa include staphylococcal enterotoxins serotypes A-E, G, and H; group A streptococcal pyrogenic exotoxins serotypes A-C and F; group A streptococcal superantigen; and staphylococcal TSST-1, which we discuss below.

All PTSAgs share common biological activities, but TSST-1 is the most divergent member of the toxin family, with less than 30[percent] amino acid homology to other family members (52-54). TSST-1 is chromosomally encoded, and the tst gene is located in a variable genetic element in S. aureus (49,52,55). The toxin is synthesized as a precursor molecule of 234 residues with the first 40 amino acids acting as a signal sequence that is cleaved to generate the mature 22 kDa toxin (49). Expression of TSST-1 depends on oxygen, temperature, pH and glucose levels, and is regulated by the S. aureus agr locus (49,51). On the basis of crystallographic analysis, TSST-1 appears structurally similar to several other PTSAgs in that the toxin consists of two distinct domains; however, unlike other family members, TSST-1 does not require a zinc cofactor (51-54). Domain A of TSST-1 (amino acid residues 1-17 and 90-194) exists as a b-grasp motif, and domain B consists of a five-stranded b-barrel motif that forms an oligosaccharide/oligonucleotide binding fold.

In general, the potent immunostimulatory properties of PTSAgs are a direct result of toxin binding to distinct regions outside the peptide binding cleft of the major histocompatibility class II molecules (expressed on the surface of **antigen** -presenting cells) and to specific Vb elements on the T-cell receptor. In particular, the domain B of TSST-1 binds primarily to the a-chain of human leukocyte **antigen** -DR1 molecules, while domain A specifically binds to human T-cell receptor Vb2 elements (51-53,56). Binding of TSST-1 to Vb2 T-cell receptor elements results in a massive proliferation of up to 20[percent] of peripheral T cells, an event that drastically skews the T-cell Vb repertoire (53,56). T cells that undergo this expansion can subsequently exist in a state of anergy or undergo apoptosis (56). Concomitant to T-cell proliferation is a massive release of both lymphocyte (interleukin IL -2, tumor necrosis factor b, gamma interferon)-derived and monocyte (IL-1, IL-6, tumor necrosis factor a)-derived cytokines (51,56). These cytokines serve as mediators of the hypotension, high fever, and diffuse erythematous rash that are characteristic of toxic-shock syndrome. Long established as a key substance in causing staphylococcal toxic-shock syndrome, TSST-1 has more recently been linked with Kawasaki syndrome, a leading cause of acquired heart disease in children in the United States (50,54).

DR. JEKYLL OR MR. HYDE?

Some of these powerful disease-causing toxins have been exploited to further basic knowledge of cell biology or for medical purposes. For example, cholera toxin and the related labiletoxin of E. coli, as well as B. pertussis toxin, have been used as biologic tools to understand the mechanism of adenylate cyclase activation and the role of cyclic AMP as a second messenger in the eukaryotic cell (57-59). Derivatives of some of these toxins, cholera toxin and labile toxin, have also been incorporated into human vaccines because of the adjuvant properties of these molecules (60,61).

Similarly, the activities of several potent cytotoxins have been harnessed as potential therapies for certain cancers. Such toxins can either be used directly in treatment or as components of immunotoxins (62-64). For example, Stx binds to the cell surface glycolipid CD77, which is expressed by B cells in certain B-cell lymphomas (65,66). This finding led to studies that showed that Stx can purge murine (and potentially human) bone marrow of malignant CD77+ B cells before an autologous bone marrow transplant (67). Other toxins that inhibit protein synthesis, such as diphtheria toxin, Pseudomonas exotoxin A, or the plant toxin ricin, are frequently engineered as the cell-killing component of immunotoxins. These "magic bullets," hybrids of the enzymatically active portion of a toxin molecule and **monoclonal** antibodies (or a receptor), are in clinical trials for the treatment of persons with B-cell lymphomas, leukemia, and bone marrow transplants.

Several clinical applications have also been found for the powerful **botulinum** neurotoxin type A (BoNT/A) (46,68). The disorders that respond to BoNT/A involve muscle hyperactivity. A minuscule amount of purified toxin injected into specific sites results in paralysis of the target muscle and ablation of the muscle spasm. Therapy must be continual since the effect of the toxin usually lasts for no more than several months. The first maladies treated with BoNT/A were eye movement abnormalities (69). However, the therapeutic value of BoNT/A has been shown for many other disorders including cervical and laryngeal dystonia, writer's cramp, hemifacial spasm, tremors, and tics (46,68). BoNT/A is also used cosmetically to reduce deep wrinkles caused by the contraction of facial muscles (70).

Another toxic bacterial product with medical applications is streptokinase, a potent plasminogen activator produced by several pathogenic streptococcal strains. The proteolytic activity of streptokinase is used to clear blocked arteries in patients who have heart attacks (71,72).

VACCINATE, DON'T PROCRASTINATE

Vaccines directed at the toxic component of bacterial pathogens have proven quite effective in preventing certain diseases. Most licensed toxoid vaccines are relatively crude, but effective, preparations. These vaccines consist of partially purified toxin preparations obtained from culture supernatants of bacteria such as C. diphtheriae, C. tetani, or B. anthracis. Formaldehyde treatment is used to detoxify the diphtheria and tetanus toxins for vaccine formulation. The anthrax vaccine contains the protective **antigen** and small amounts of the lethal factor and edema factor toxins. The current **botulinum** vaccine is an investigational drug composed of crude preparations of five **botulinum** toxoids and is distributed by the Centers for Disease Control and Prevention to researchers that work with the toxin or organism. Acellular pertussis vaccines that contain pertussis toxoid, alone or as one of several components, are as effective as killed whole-cell vaccines but less reactogenic (73); such vaccines have recently been approved for use in infants as well as older children.

New vaccines aimed at toxins are in various stages of development: research and development, preclinical, phase I, phase II, or phase III (74). The next generation of toxoid vaccines falls into three general categories: purified toxoids that have been inactivated by chemical or genetic means; live, attenuated strains of the causative agent that produce a genetically derived toxoid; or live, attenuated unrelated bacterial vector strains, such as V. cholerae or Salmonella, that produce the target toxoid. Examples of each of these approaches and progress in development of specific toxoid vaccines are described annually in the Jordan Report (74).

Antitoxins raised against diphtheria, tetanus, and **botulinum** toxoids have also been used for many years to treat seriously ill patients. Antiserum specific for the Stx toxins produced by E. coli O157:H7 and other STEC is under development for the treatment and prevention of hemolytic uremic syndrome, a life-threatening sequela of these infections.

SUMMARY

Microbial toxins capable of interrupting or hyperstimulating many essential functions and pathways of eukaryotic cells have evolved along with the carrier bacterium. Presumably these toxins confer some benefit to the

bacterium, either during a stage of the host-parasite interaction or in some environmental niche encountered by the bacterium. Certain bacterial toxins act on the target cell surface to irreparably damage the cell membrane or alter normal cellular signal transduction. Other toxins exhibit enzymatic activity once the molecule has gained access to the cytoplasm of the sensitive cell by endocytosis. Yet other bacterial toxins act by either turning off or locking on a normal host cell function.

Although detrimental to the susceptible host during an infection, the activities of several bacterial toxins have been exploited as probes of eukaryotic cellular pathways and for medicinal applications. Thus, research on a microbial toxin produced by an established, emerging, or reemerging pathogen is likely to yield novel information about the role of that toxin in disease as well as the properties of host cells that are subverted by the toxin.

Added material

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Table. Characteristics of bacterial toxins(FNa)

FOOTNOTES

a Abbreviations: CNF, cytotoxic necrotizing factor; LT, heat-labile toxin; ST, heat-stable toxin; CLDT, cytolethal distending toxin; EAST, enteroaggregative E. coli heat-stable toxin; TCR, T-cell receptor; MHC II, major histocompatibility complex class II; **MAPKK** , mitogen-activated protein kinase kinase; VAMP, vesicle-associated membrane protein; SNAP-25, synaptosomal-associated protein; UTI, urinary tract infection; HC, hemorrhagic colitis; HUS, hemolytic uremic syndrome; PC, antibiotic-associated pseudomembranous colitis; SSS, scalded skin syndrome; SF, scarlet fever; TSS, toxic-shock syndrome.

b Yes, strong causal relationship between toxin and disease; (yes), role in pathogenesis has been shown in animal model or appropriate cell culture;?, unknown.

c Other diseases are also associated with the organism.

d Toxin is also produced by other genera of bacteria.

Figure 1. Diagrammatic representation of the mode of action of several bacterial toxins. A. Damage to cellular membranes by Staphylococcus aureus a-toxin. After binding and oligomerization, the stem of the mushroom-shaped a-toxin heptamer inserts into the target cell and disrupts membrane permeability as depicted by the influx and efflux of ions represented by red and green circles. B. Inhibition of protein synthesis by Shiga toxins (Stx). Holotoxin, which consists of an enzymatically active (A) subunit and five binding (B) subunits, enters cells through the globotriasylceramide (Gb3) receptor. The N-glycosidase activity of the A subunit then cleaves an adenosine residue from 28S ribosomal RNA, which halts protein synthesis. C. Examples of bacterial toxins that activate secondary messenger pathways. Binding of the heat-stable enterotoxins (ST) to a guanylate cyclase receptor results in an increase in cyclic GMP (cGMP) that adversely effects electrolyte flux. By ADP-ribosylation or glucosylation respectively, the C3

exoenzyme (C3) of Clostridium **botulinum** and the Clostridium difficile toxins A and B (CdA & CdB) inactivate the small Rho GTP-binding proteins. Cytotoxic necrotizing factor (CNF) of E. coli and the dermonecrotic toxin (DNT) of Bordetella species activate Rho by deamidation.

Figure 2. Ribbon crystal structures of Shigella dysenteriae Shiga toxin (20), Escherichia coli heat-labile toxin I (LT-I) (21), and pertussis toxin (22). The Shiga toxin figure was contributed by Marie Frasier. The LT-I and pertussis figures were contributed by Ethan Merritt. The figures were drawn in MOLSCRIPT (75).

Figure 3. The effect of cytotoxic necrotizing factor type 1 (CNF1) on eukaryotic cells. A. HEp-2 cells, magnification 10X. B. HEp-2 cells intoxicated with CNF1, magnification 10X.

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Immunological characterization of papain-induced fragments of Clostridium botulinum type A neurotoxin and interaction of the fragments with brain synaptosomes

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To clarify the role that the **carboxyl** -terminal portion of the **heavy chain** plays in the binding of the neurotoxin to synaptosomes, attempts were made to obtain a **carboxyl** -terminal portion of the **heavy chain** by treating type A neurotoxin with papain. In addition, we scrutinized the **antigenic** structure of the neurotoxin and its fragments from **epitopes** recognized by MAbs. This report also describes the properties of the type A neurotoxin-binding site on the synaptosome membrane

English Descriptors: Neurotoxin; Enzymatic digestion; Papain; Fragments; **heavy** -Peptide **chain** ; Characterization; Binding site; Synaptosome; Ganglioside; Immunoblotting assay; **Monoclonal** antibody; Brain; Mouse; In vitro; Toxin; Enzyme; Glycolipid; Host agent relation; Clostridium **botulinum**

Broad Descriptors: Rodentia; Mammalia; Vertebrata; Clostridiaceae; Clostridiales; Bacteria; Rodentia; Mammalia; Vertebrata; Clostridiaceae; Clostridiales; Bacterie; Rodentia; Mammalia; Vertebrata; Clostridiaceae; Clostridiales; Bacteria

French Descriptors: Neurotoxine; Degradation enzymatique; Papain; Fragment; Chaine peptidique lourde; Caracterisation; Site fixation; Synaptosome; Ganglioside; Methode immunoblotting; Anticorps **monoclonal** ; Encephale; Souris; In vitro; Toxine; Enzyme; Glycolipide; Relation hote agent; Clostridium **botulinum**

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Introduction

Chronic granulomatous disease (CGD) is a rare inherited disorder of the reduced nicotinamide dinucleotide phosphate (NADPH) oxidase complex in which phagocytes are defective in generating the microbicidal reactive oxidant superoxide anion and its metabolites, hydrogen peroxide, hydroxyl anion, and hypohalous acid. As a result of the defect in this key host defense pathway, CGD patients suffer from recurrent life-threatening bacterial and fungal infections (Figure 1). CGD is also characterized by abnormally exuberant inflammatory responses leading to granuloma formation, such as granulomatous enteritis, genitourinary obstruction, and poor wound healing and dehiscence (Figure 2). CGD affects approximately 1 in 200,000 persons.

(Figures 1-2 ILLUSTRATION OMITTED)

CGD was first described by Good et al (32, 44) as a fatal granulomatous disease of childhood. The authors noted the similarity with other granulomatous diseases such as scrofula, Wegener granulomatosis, brucellosis, tularemia, cat scratch disease, sarcoidosis, and Hodgkin disease. However, they recognized the combination of recurrent suppurative infections and inflammatory complications occurring in childhood as a distinct clinical syndrome. The granulomata occurred in the liver, lungs, spleen, and lymph nodes. In early clinical reports, CGD was only diagnosed in boys, but over several years, it became appreciated that both X-linked and autosomal recessive modes of inheritance exist (32, 44, 57, 227).

In the 1960s, classic studies established CGD as a disease of phagocytes. Neutrophils from CGD patients demonstrate normal phagocytic activity in vitro, but bactericidal activity against Staphylococcus aureus is markedly impaired (182, 321, 322). In addition, CGD neutrophils fail to show an increase in oxygen consumption and hydrogen peroxide formation in response to phagocytosis of latex particles (182).

The diagnosis of CGD is based on a compatible clinical history and demonstration of a defective respiratory burst. Several methods detect the production of reactive oxidants. The nitroblue tetrazolium (NBT) method relies on the intracellular reduction of NBT by superoxide anion to a blue formozan precipitate that can be seen microscopically. More sensitive methods rely on the reaction of oxidants with specific chemiluminescent and fluorescent probes (discussed later).

Over the past 10 years, the membrane-bound and cytoplasmic components of the phagocyte NADPH oxidase have been identified, cloned, and sequenced and their mechanisms of assembly into a functional enzyme complex have been characterized. CGD is a genetically heterogenous disease caused by mutations in any of 4 structural components of NADPH oxidase, including the membrane-bound glycoprotein (gp91.sup.phox) (phagocyte oxidase) and (p22.sup.phox) and the cytoplasmic components (p47.sup.phox) and (p67.sup.phox). Extensive databases listing the locations of mutations causing CGD have been published (76, 77, 175, 329, 331). More recently, mouse knockout models of (gp91.sup.phox) and (p47.sup.phox) have been created, and gene therapy trials in CGD patients are underway.

The role of reactive oxidants in a variety of normal and pathologic

states, including intracellular signaling (15, 27, 34, 36, 46, 134, 138, 161, 183, 186, 197, 264, 267, 308, 312, 341, 348, 365, 366, 388), apoptosis (8, 37, 53, 74, 131, 161, 177, 202, 221,242, 343, 389, 391,405, 418), sepsis (149, 158, 382, 383, 392), the respiratory distress syndrome (36, 52, 129, 224, 383), malignant transformation (163, 186, 306, 341, 422), ischemic reperfusion injury (7, 12, 51, 183, 303, 414), and atherosclerosis (10, 34, 71, 99, 150, 296, 417), has generated intense interest in the NADPH oxidase. In this review, we will discuss the basic science and biochemistry of the NADPH oxidase as well as clinical and therapeutic developments in CGD.

NADPH Oxidase

Overview

It has been known since the 1930s that a dramatic increase in neutrophil oxygen consumption, termed the "respiratory burst" occurs following phagocytosis. Initially, this respiratory burst was attributed to increased mitochondrial glucose oxidation. However, this increase in oxygen consumption is insensitive to mitochondrial inhibitors (336, 346). The respiratory burst following phagocytosis is restricted to the membrane fraction of neutrophil lysates and accompanied by an increase in hexose monophosphate shunt activity (335). Stimulated neutrophils oxidize NADPH by a reaction that produces hydrogen peroxide (190). The clinical significance of these findings was recognized when it was discovered that phagocytes from CGD patients do not generate the respiratory burst (182, 321).

The phagocyte NADPH oxidase functions to rapidly generate superoxide anion by transferring electrons from NADPH to molecular (O.sub.2) in response to physiologic stimuli such as phagocytosis (Figure 3). The cytochrome, composed of (gp91.sup.phox) and (p22.sup.phox), is embedded in membranes. In neutrophils, approximately 85% of the cytochrome is in the membranes of specific granules or gelatinase-containing granules, and the remainder is present in the plasma membrane and in secretory granules (39, 41, 209). In neutrophils, the activated oxidase may assemble exclusively within the granules, with release of extracellular superoxide anion occurring only following fusion of the granules with the plasma membrane (220). The NADPH-binding site is located on the cytoplasmic side of membranes. Upon activation of the oxidase, the cytoplasmic subunits (p47.sup.phox), (p67.sup.phox) and (p40.sup.phox) appear to translocate en bloc to the membrane-bound cytochrome. NADPH is oxidized to (NADP.sup.+), and electrons are transported down a reducing potential gradient to flavin adenine dinucleotide (FAD) and then possibly to 2 nonidentical heme groups. On the vacuolar or extracellular side of the membrane, the final step in the electron transport chain occurs when oxygen accepts an electron and is converted to superoxide anion. The net equation involves the reduction of 2 molecules of (O.sub.2) to 2 molecules of superoxide anion (MATHEMATICAL EXPRESSION NOT REPRODUCIBLE IN ASCII) at the expense of 1 molecule of NADPH.

(MATHEMATICAL EXPRESSION NOT REPRODUCIBLE IN ASCII)

(Figure 3 ILLUSTRATION OMITTED)

Superoxide, a relatively weak microbicidal oxidant, is metabolized to the more toxic hydrogen peroxide by superoxide dismutase. Hydrogen peroxide in turn can be converted to hypohalous acid (bleach in the neutrophil) by myeloperoxidase, and to hydroxyl anion. Activated human neutrophils convert (MATHEMATICAL EXPRESSION NOT REPRODUCIBLE IN ASCII) into the proinflammatory oxidants (NO.sub.2)Cl and (multiplied by) (NO.sub.2) through a myeloperoxide-dependent pathway. Inducible nitric oxide synthase (iNOS) and myeloperoxidase colocalize in the primary granules of neutrophils, suggesting that the products of these pathways, (NO.sub.2)Cl, (multiplied by) (NO.sub.2), and superoxide anion, may commingle in the phagocytic vacuole, forming novel host defense pathways (116, 126). Reactive oxygen and nitrogen intermediates have synergistic bactericidal activity in a variety of in vitro systems (211, 213, 295). Possible molecular targets of these species include genomic DNA, electron transport, and sulfhydryl groups of proteins and nonprotein targets (295).

Cytochrome (b.sub.558)

Segal and Jones (354, 355) identified a cytochrome within phagocytic vacuoles that is either absent or abnormal in cell homogenates from CGD patients. It is referred to as cytochrome (b.sub.558) because of its peak in infrared absorbance, but it is also known as cytochrome (b.sub.-245) because of its unusually low midpoint potential (- 245 mv), making it thermodynamically feasible to function as an electron carrier in the

reduction of oxygen to superoxide anion (79). Cytochrome b is present at ~100 pmol/mg protein in normal neutrophils and in monocytes, macrophages, eosinophils, and B lymphocytes (352). In a European study, the cytochrome was undetectable in all 19 men with X-linked CGD, whereas heterozygous female carriers had a reduced concentration of the cytochrome (352). The concentration of cytochrome b in neutrophil preparations correlated with the proportion of cells with intact oxidase activity. In contrast, 8 patients with autosomal recessive CGD had normal cytochrome content, but impaired superoxide production (352).

Membranes from stimulated neutrophils rapidly reduce NADPH under aerobic but not anaerobic conditions in parallel with superoxide production, whereas membranes from unstimulated neutrophils do not (80). The Km value for NADPH is ~45 (micro)M (80).

Royer-Pokora et al (340) identified the gene for X-linked CGD at Xp21.1 by positional cloning in 2 patients with interstitial deletions of Xp21 leading to Duchenne muscular dystrophy (Online Mendelian Inheritance in Man (OMIM) 310200(*)), the Mcleod syndrome (OMIM 314850), and X-linked CGD (22, 340). They enriched cDNA for Xp21 transcripts by subtractive hybridization with RNA from a CGD patient with an Xp21 deletion, allowing them to identify a gene expressed only in phagocytes that was absent or abnormal in patients with X-linked CGD.

In complementary studies, Parkos et al (301) and the Segal group (351,398) purified and characterized the polypeptide constituents of cytochrome b. The subunits, 22 kDa and 91 kDa proteins, referred to as the (Alpha) and (Beta) components, respectively, remain closely associated with each other and with the heme spectrum during purification, being separated only by denaturation (301,351). Neither protein was present in neutrophils from 5 patients with X-linked CGD, but both were present in 2 patients with autosomal recessive CGD (351). Antibody raised against the polypeptides showed no binding to neutrophil preparations from X-linked CGD patients (103, 301, 351). Teahan et al (398) showed that the N-terminal amino acid sequence of the 91 kDa subunit corresponded to the 5' region of the X-linked CGD gene identified by Royer-Pokora et al (340).

The gene for (gp91.sup.phox), CYBB (cytochrome b, (Beta)-subunit), encodes a 570 amino acid protein which is heavily glycosylated with N-linked carbohydrates, primarily N-acetyl glucosamine and galactose (166). The gene for (p22.sup.phox), CYBA (cytochrome b, (Alpha)-subunit), encodes a 195 amino acid protein which is not glycosylated (302). In contrast to the predominantly myeloid expression of (gp91.sup.phox), (p22.sup.phox) mRNA is expressed in many cell types (302). The N-terminus of (p22.sup.phox) is hydrophobic and may contain membrane-spanning domains. Typically, defects in either the light or **heavy chain** lead to absence of both components of the cytochrome from phagocyte membranes, suggesting that the subunits are required to stabilize each other within the membrane (104). The majority of cases of CGD are cytochrome-negative arising from defects in either the X-linked (gp91.sup.phox) or the autosomal (p22.sup.phox) gene.

An FAD-binding protein is necessary for NADPH oxidase activity (19). It was postulated that the flavoprotein binds NADPH and transfers electrons to the cytochrome which in turn provides the reducing equivalents to generate superoxide anion from oxygen. Rotrosen et al (339) and Segal et al (356) showed independently that cytochrome (b.sub.558) alone was capable of binding NADPH and FAD, and when combined in cell-free systems with purified (p47.sup.phox), (p67.sup.phox), and Rac proteins, generated superoxide anion (339, 356). Purified cytochrome (b.sub.558) can generate superoxide in the absence of cytoplasmic components, further suggesting that the cytochrome is sufficient to drive electron transport within the NADPH oxidase (222, 223). The cytochrome binds heme and FAD in a molar ratio of 2:1 (356). The heme group midpoint potentials are thermodynamically feasible to function as intermediates in electron transport through the membrane (81). However, Babior (18) suggests that the rate of reduction of the cytochrome b heme by NADPH is too slow for it to function as an obligatory intermediate in electron transfer leading to superoxide production. (p67.sup.phox) may also contain an NADPH-binding site, but its functional relevance is unknown (376).

Using the crystal structure of ferredoxin reductase from spinach leaves, Taylor et al (397) constructed a 3-dimensional model of the homologous **C - terminal** region of (gp91.sup.phox) in which the **C - terminal** half binds NADPH and FAD and functions as the proximal electron

transporter of the NADPH oxidase complex. Immunochemical studies confirmed the cytoplasmic location of the **C - terminus** of (gp91.sup.phox) (337, 427). (gp91.sup.phox) contains a stretch of 20 amino acids (residues 484-504) which lie over the proposed NADPH binding cleft, and may have to be displaced by cytoplasmic components to allow NADPH binding (397, 401).

There are heme groups, N-linked glycosylation sites, and a proton conduction channel within the N-terminus of (gp91.sup.phox) (435). The N-terminus is hydrophobic with 4-6 predicted transmembrane helices. Finegold et al (136) proposed coordination of the 2 heme groups by 4 critical histidine residues in the N-terminal half of (gp91.sup.phox), based on the similarity between NADPH oxidase and the yeast plasma membrane iron reductase FRE1. In this model, 1 heme group is coordinated between the imidazole rings of histidine residues 101 and 209, and the other between histidine residues 115 and 222 of (gp91.sup.phox). The hemes are situated between transmembrane helices and are predicted to lie 1 on top of the other and perpendicular to the plane of the membrane, as in other organelle cytochromes b that function in transmembrane electron transport (344, 425). Three X-CGD patients without detectable heme spectra or (gp91.sup.phox) protein have been identified with single mutations at histidines 101, 209, and 222, supporting the critical role of these highly conserved histidine residues in heme binding (136, 329, 331).

Superoxide anion generation by the NADPH oxidase is associated with an efflux of protons through an arachidonic acid-activatable (H.sup.+) channel located in the N-terminal portion of (gp91.sup.phox) (168, 169). This (H.sup.+) conduction pathway is necessary to compensate for the depolarization of the membrane potential during oxidase activation and to avoid a rapid fall in intracellular pH. His-115 is critical in (H.sup.+) conduction and also functions in binding to 1 of the heme groups in (gp91.sup.phox) (136, 168). Seligmann and Gallin (364) first noted that the normal changes in membrane potential in neutrophils elicited by chemoattractants are either reduced or absent in neutrophils from CGD patients. CGD neutrophils are also defective in intracellular acidification, and exogenous acidification of the extracellular fluid restores normal membrane potential responses to the chemoattractant leukotriene (B.sub.4) (5). Geiszt et al (156) observed that calcium influx into normal neutrophils via the capacitative or store-regulated pathway is inhibited by the membrane depolarization accompanying superoxide generation by the NADPH oxidase, and that calcium influx is altered in CGD neutrophils. Thus the NADPH oxidase, through its regulation of membrane potential, pH balance, and calcium homeostasis, may have roles in cell signaling independent of its microbicidal function.

Normal (gp91.sup.phox) cDNA expressed in vitro without glycosylation yields a product of ~58 kDa (434). Progressive glycosylation produces a 76-90 kDa product. In 2 patients with (p22.sup.phox) CGD, a 65 kDa precursor of (gp91.sup.phox) was identified in the membrane fraction, representing incomplete glycosylation (318). Restoration of (p22.sup.phox) expression in B-cell lines from these patients resulted in a functional NADPH oxidase and expression of normally glycosylated (gp91.sup.phox), suggesting that full glycosylation of (gp91.sup.phox) is dependent on its interaction with (p22.sup.phox) (318). The 65 kDa protein is undetectable in normal mature neutrophils, perhaps because it is rapidly degraded or glycosylated to the mature (gp91.sup.phox) product (434). Similarly, another CGD patient's mutation leading to an inappropriate glycosylation of asparginine at position 154 of (gp91.sup.phox) was associated with loss of (p22.sup.phox), suggesting that appropriate glycosylation of (gp91.sup.phox) is a requirement for stabilization of the cytochrome heterodimer (415).

To delineate more clearly the sequential interactions between the 65 kDa precursor, (gp91.sup.phox), and (p22.sup.phox), (gp91.sup.phox) cDNA was constitutively expressed in the PLB-985 myeloid cell line from an X-CGD patient and the cellular localization of these proteins evaluated (434). (p22.sup.phox) only co-sedimented with the 91 kDA form of (gp91.sup.phox). Treatment of cells with the heine synthesis inhibitor, succinyl acetone, inhibited (gp91.sup.phox) (p22.sup.phox) dimerization, suggesting that heme insertion into (gp91.sup.phox) is required to stabilize the dimer (434).

Cytosolic components of the NADPH oxidase

When monocytes of an X-linked cytochrome b-negative ((X91.sup.0)) CGD patient were fused with monocytes from an autosomal recessive cytochrome b-positive ((A.sup.+)) patient, the heterologous hybrids were capable of

generating superoxide production (165). Complementation did not occur when homologous hybrids were used. A third form of CGD was recognized using this method when monocytes from a patient with autosomal recessive cytochrome-negative ((A.sup.-)) CGD fused with either monocytes from (X91.sup.0) or (A.sup.+) monocytes yielded hybrids with normal oxidase function (419). At the time of these early studies, neither the genes nor the protein products of the NADPH oxidase were characterized, but 3 forms of CGD were documented: X-linked, (A.sup.-) (autosomal recessive, cytochrome negative), and (A.sup.+) (autosomal recessive, cytochrome positive).

Since the work of the Rossi group (335) in the 1960s, it has been known that NADPH oxidase activity is confined to the particulate membrane fraction of neutrophils, but oxidase activity only occurs if neutrophils have been preactivated (for example, with opsonized zymosan). Arachidonic acid and other long-chain fatty acids stimulate NADPH oxidase activity in intact neutrophils and in cell-free preparations from unstimulated human neutrophils in which the pellet and supernatant fractions are combined, but not from membrane fractions alone, indicating a need for cytosolic factors (21, 45, 82, 84, 85, 261). Prestimulation of neutrophils with N-formyl peptide or phorbol myristate acetate (PMA) obviates the cytosol requirement. These results were similar to those generated in other species (45, 172).

Patients with either the X-linked or (A.sup.-) forms of CGD have normal cytosolic activity in cell-free systems, whereas in patients with the (A.sup.+) form of CGD, cytosolic factor activity is impaired, but membrane oxidase activity can be activated by control cytosol and arachidonate (85). Therefore, these studies indicate that both cytosolic and membranous components are necessary for oxidase function, and that CGD may arise from defects in either location.

Nunoi et al (289) and Volpp et al (407) identified 2 forms of autosomal CGD in which either a 47 kDa (termed neutrophil cytosolic factor-1 (NCF-1)) or a 67 kDa (termed neutrophil cytosolic factor 2 (NCF2)) protein is deficient. Purified NCF-1 added to cytosolic fractions restores oxidase activity in cell-free systems from most (A.sup.+) CGD patients; in patients with (A.sup.+) CGD, NCF-2 is required to restore cytosolic activity (289). Lack of NCF-1 function is associated with absence of the 47 kDa protein by immunoblot analysis, and NCF-2 deficiency is associated with absence of the 67 kDa protein (289, 407). In a study of 25 (A.sup.+) CGD patients, 22 patients lacked the 47 kDa protein and 3 patients lacked the 67 kDa component (67).

Soon after their functional identification, the genes encoding (p47.sup.phox) (NCF-1) and (p67.sup.phox) (NCF-2) were cloned and sequenced (235, 246, 408). Recombinant proteins reconstituted neutrophil cytosolic activity from (A.sup.+) CGD patients in cell-free systems, (p47.sup.phox) is a 390 amino acid highly basic protein (pI = 10.4) with an arginine and serine rich **C - terminus** with potential phosphorylation sites (246, 408). (p67.sup.phox) is slightly acidic (pI ~ 6) with 526 amino acids (86, 235). Both proteins contain 2 segments with structural similarity to portions of the noncatalytic domains of src-related tyrosine kinases (known as SH3 domains) (235, 246, 408). These domains bind noncovalently to proline-rich targets, mediating intra- and intermolecular peptide interactions. Activation of NADPH oxidase involves translocation of the (p47.sup.phox) and (p67.sup.phox) subunits and the low molecular weight GTP binding protein Rac to the membrane where they assemble with cytochrome b (Figure 4). Protein-protein interactions involving SH3 domains are critical to the assembly of the oxidase. Another cytosolic component of the NADPH oxidase, (p40.sup.phox), associates and translocates with (p47.sup.phox) and (p67.sup.phox), and may have a downregulatory role (discussed later). NADPH oxidase components and CGD genotypes and cellular phenotypes are outlined in Tables 1 and 2.

(Figure 4 ILLUSTRATION OMITTED)

TABLE 1. Characteristics of NADPH oxidase components in chronic granulomatous disease

	(gp91.sup.phox)
Frequency	70%
Gene	
OMIM	306400

Name	CYBB
Location	Xp21.1
Exons	13
Transcriptional regulators(*)	PU.1, CP1, BID CDP, IRF-1, ICSBP
Protein	
Amino acids	570
Post-translational modification	N-glycosylation
Tissue expression	Phagocytes((dagger)), B lymphocytes
Cell location	
Resting	Membrane
Activated	Membrane
	(p22.sup.phox)
Frequency	5%
Gene	
OMIM	233690
Name	CYBA
Location	16q24
Exons	5
Transcriptional regulators(*)	?
Protein	
Amino acids	195
Post-translational modification	Phosphorylation
Tissue expression	Ubiquitous B lymphocytes
Cell location	
Resting	Membrane
Activated	Membrane
	(p47.sup.phox)
Frequency	20%
Gene	
OMIM	233700
Name	NCF1
Location	7q11.23
Exons	9
Transcriptional regulators(*)	PU.1
Protein	
Amino acids	390
Post-translational modification	Phosphorylation
Tissue expression	Phagocytes((dagger)), B lymphocytes
Cell location	
Resting	Cytoplasm
Activated	Membrane
	(p67.sup.phox)
Frequency	5%
Gene	
OMIM	233710
Name	NCF2
Location	1q25

Exons	16
Transcriptional regulators(*)	?
Protein	
Amino acids	526
Post-translational modification	Phosphorylation
Tissue expression	Phagocytes((dagger)), B lymphocytes
Cell location	
Resting	Cytoplasm
Activated	Membrane

(*) Data exist that support a role for each of these transcription factors in regulating the expression of the (gp91.sup.phox) gene. Putative functions of each of these factors and possible interactions among transcription factors are discussed in the text.

((dagger)) Recent studies suggest that NADPH oxidase components or another oxidant-producing flavocytochrome may be expressed in non-myeloid and non-lymphoid cells, such as fibroblasts, smooth muscle, endothelial, and tumor cells (34, 186, 259, 296, 297).

TABLE 2. CGD genotype and cellular phenotype

Genotype	Flavocytochrome(*)
X-linked	
(X91.sup.0)	Not detectable
(X91.sup.+)	Normal quantity, defective (gp91.sup.phox)
(X91.sup.-)	(down arrow) Quantity
Autosomal recessive	
(A.sup.-) (p22.sup.phox)	Not detectable
(A.sup.+) (p22.sup.phox)	Normal quantity, defective (gp22.sup.phox)
(A.sup.+) (p47.sup.phox)	Normal
(A.sup.+) (p67.sup.phox)	Normal

Genotype	(p47.sup.phox) ((dagger))	(p67.sup.phox) ((dagger))
X-linked		
(X91.sup.0)	Normal	Normal
(X91.sup.+)	Normal	Normal
(X91.sup.-)	Normal	Normal
Autosomal recessive		
(A.sup.-) (p22.sup.phox)	Normal	Normal
(A.sup.+) (p22.sup.phox)	Normal	Normal
(A.sup.+) (p47.sup.phox)	Not detectable	Normal
(A.sup.+) (p67.sup.phox)	Normal	Not detectable

(*) Detected by spectral analysis or immunoblotting.

((dagger)) In the vast majority of (p47.sup.phox) and (p67.sup.phox) CGD patients, the respective protein is not detectable by immunoblotting. When the defective protein is detectable, the designation (p47.sup.phox+) or (p67.sup.phox+) can be used.

Small G proteins

Rac: The Ras superfamily of small (~21 kDa) GTP-binding proteins regulates diverse signal transduction pathways including cell cycling, cellular differentiation, mitogenesis, and assembly and disassembly of the actin cytoskeleton in response to extracellular signals (395). Ras proteins cycle between active GTP-bound and inactive GDP-bound states. Numerous guanine nucleotide exchange factors (GEF) and GTPase-activating proteins (GAP) that regulate the activity of Ras proteins have been identified (395).

Rac is required for oxidase function (2, 218, 226). Cell-free oxidase systems require added GTP, whereas GDP analogues inhibit oxidase activation (87, 217, 311, 362). Post-translational attachment of 15-20 carbon isoprenoid moieties are common in the Ras-superfamily of GTPases, and inhibitors of isoprenylation reduce the rate of superoxide generation (217). Abo et al (2) purified Rac1 from guinea pig macrophage cytosol along

with the GDP-dissociation inhibitor rhoGDI.

Knaus et al (217) purified a second GTP-binding protein, Rac2, from human neutrophil cytosol that was capable of stimulating oxidase activity in cell-free systems. When Epstein-Barr virus (EBV)-transformed B-lymphocytes, which express Rac1 and Rac2 genes, were treated with Rac antisense oligonucleotides, superoxide production was inhibited (110). Both Rac1 and Rac2 belong to the Rho subfamily of Ras proteins, and have 92% amino acid sequence homology. Rac1 is ubiquitously expressed, whereas Rac2 is only expressed in myeloid cells (100, 217). Neutrophil cytosol contains predominantly Rac2, almost all of which is complexed with rhoGDI in the inactive state. In both whole cells and in cell-free assays, activation of NADPH oxidase is associated with dissociation of Rac from rhoGDI (3). The rate of superoxide production is increased by exogenous GTP, but is inhibited by GDP, implicating rhoGDI as the GDP dissociation inhibitor of Rac. Release of Rac from this complex, enabling it to bind to GTP, is necessary for NADPH oxidase activation (3).

Deikman et al (101) and Prigmore et al (319) demonstrated that Rac binds exclusively to (p67.sup.phox) in a GTP-dependent manner. Mutational analysis of (p67.sup.phox) identified residues 170-199 as the critical minimal regions for Rac binding, but residues 1-192 are required for full binding activity (6). Deletion of the (p67.sup.phox) **C - terminus** (residues 193-526), the **C - terminal** SH3 domain (residues 470-526), or the proline-rich motif (residues 226-236) increases binding to Rac 1, suggesting that these regions of (p67.sup.phox) impede Rac binding (6).

The N-terminus of Rac is the effector-specifying domain for oxidase activation (226). Both Rac1 and Rac2 bind to (p67.sup.phox) with a 1:1 stoichiometry, with a Kd of 120 and 60 nM, respectively (287). (p67.sup.phox) binds with the Rac effector region (residues 26-45), but not at the insert region (residues 124-135), a stretch of amino acids not found in other members of the Ras family. NADPH oxidase activity is inhibited by mutations in either region, indicating that both the effector and insert regions of Rac are required for oxidase function (287).

Immunoelectron microscopy shows that NADPH oxidase components and Rac colocalize in distinct clusters on the cytoplasmic surface of the membrane in activated, but not in unstimulated, neutrophils (427). Heyworth et al (173) showed that Rac2 translocation to the membrane is dependent on the flavocytochrome (that is, Rac2 translocation was reduced in X-CGD neutrophils), but was independent of the (p47.sup.phox) and (p67.sup.phox) components. These data suggest that Rac and the cytosolic phox components bind to different sites on the flavocytochrome. Rac, in part through its interaction with (p67.sup.phox), is necessary to stabilize the complex of cytoplasmic phox proteins with the flavocytochrome.

Leusen et al (238) identified a CGD patient who was a compound heterozygote for the (p67.sup.phox) gene; 1 allele had a large deletion and the other had an inframe deletion causing loss of lysine 58. The mutant lys(Delta)58 (p67.sup.phox) protein failed to bind to Rac1, to translocate to the membrane following stimulation, or to support oxidase activity (238).

Several other targets of Rac and other Ras proteins have been identified (395). Knaus et al (219) identified 65 kDa and 68 kDa kinases as Rac targets in human neutrophils, termed p21 activated kinases (PAKs). Both PAKs bind specifically to RacGTP and autophosphorylate. Rapid (30-60 sec) and transient activation of PAK 1 (65 kDa) and PAK 2 (68 kDa) occur following stimulation with the chemoattractant N-formyl peptide. Native and recombinant PAK 1 and 2 also phosphorylate (p47.sup.phox) in the presence of Rac-GTP, which is important since (p47.sup.phox) is extensively phosphorylated at its **C - terminus** during oxidase activation in vivo (132,353). (p67.sup.phox) is PAK-phosphorylated adjacent to its binding site with Rac (6).

Because Rac1 and Rac2 are coexpressed in myeloid cells and both are capable of supporting oxidase function in cell-free systems, it has been unclear whether they have redundant functions. Rac2 constitutes (is greater than) 96% of the Rac protein in human neutrophils (173) and (p67.sup.phox) binding with Rac2 is 6-fold greater than with Rac1 in the yeast 2-hybrid system (109), suggesting that Rac2 may have the predominant role in oxidase activation. Targeted Rac2-deficient mice are deficient in chemotaxis, F-actin generation, and p38 and p42/p44 **MAP** kinase activation (327). In vivo, Rac2-deficient mice have neutrophilia, reduced experimental peritoneal inflammatory exudate, and increased mortality from experimental

Aspergillus fumigatus infection (327). Superoxide production from PMA-stimulated bone marrow-derived neutrophils was reduced by ~80% in Rac2-deficient mice compared with wildtypes. In contrast, superoxide production from thioglycollate-elicited peritoneal exudate cells was similar between the 2 groups. Prestimulation of Rac2-deficient bone marrow neutrophils with tumor necrosis factor-(Alpha) (TNF-(Alpha)) prior to PMA stimulation partially corrected the deficiency in superoxide production. Therefore, Rac2 plays a key role in oxidase activity, but additional pathways support oxidase activity in certain settings (327).

Caron and Hall (56) identified 2 pathways of phagocytosis controlled by different Rho-related members of the Ras family. Phagocytosis via the immunoglobulin receptors (Fc(Gamma)Rs) is mediated by Cdc42 and Rac, whereas phagocytosis via the complement receptor (CR3) is mediated by Rho. Activation of either receptor is associated with reorganization of F-actin in the cytoskeleton. IgG-dependent phagocytosis is accompanied by pseudopod extension, membrane ruffling, production of arachidonic acid metabolites and cytokines, and NADPH oxidase activation, whereas complement-dependent phagocytosis occurs in the absence of these events (56). Therefore, Rac links IgG-dependent phagocytosis with cytoskeletal reorganization and NADPH oxidase activation (56).

Rap1A: Rap1A is another member of the Ras family that copurifies and immunoprecipitates with cytochrome (b.sub.558) of neutrophils (323). Rap1A and cytochrome (b.sub.558) form stoichiometric complexes, with Rap1A-GTP binding more tightly to the cytochrome than Rap1A-GDP (38). Phosphorylation of Rap1A at a C- **terminal serine** residue by cAMP-dependent protein kinase abolishes binding with the cytochrome (38). Immunodepletion of Rap1A from neutrophil cytosol results in the loss of cell-free NADPH oxidase activity (118), suggesting that translocation of cytosolic Rap1A to cytochrome (b.sub.558) is important for oxidase activity and that phosphorylation of Rap1A may inhibit oxidase activity by preventing association with the cytochrome.

Gabig et al (148) proposed that Rac 1/2 and Rap1A regulate NADPH oxidase activity at distinct sites. Rac, through binding with (p67.sup.phox) and interaction with the cytoskeleton, may bring the cytosolic components in close proximity to the membrane-bound cytochrome. Rac may also activate the cytosolic components via PAKs. Phosphorylation of specific residues of (p47.sup.phox) may induce conformational changes, thereby exposing domains required for interaction with other oxidase components. To date, no mutations in Rac or Rap1A components of the oxidase have been identified.

Rap1A functions in a dynamic cycle in which exchange between the GTP- and GDP-bound forms regulate oxidase activity. Rap1A is transiently activated (as measured by accumulation of Rap1A in its GTP-bound form) by N-formyl peptide, platelet aggregating factor, GM-CSF, and IgG coated particles, and activation is independent of the respiratory burst (250).

NADPH oxidase assembly and activation

Activation of the NADPH oxidase is a complex process involving multiple signal transduction events. In the inactive state, the cytosolic subunits are held together by precise interactions involving SH3 domains and proline-rich motifs. Assembly of the activated complex involves conformational changes that "unmask" SH3 binding sites and permit binding between the cytosolic subunits and the flavocytochrome. (p47.sup.phox) is phosphorylated at multiple sites in a regulated sequential fashion, suggesting that changes in electrostatic interactions have an important role in activation. The cytosolic subunits (p47.sup.phox), (p67.sup.phox), and (p40.sup.phox) translocate as a unit to the plasma membrane where they associate with hydrophilic regions of the flavocytochrome. In the inactive state, Rac is bound to rhoGDI, which prevents dissociation of GDP from Rac. Rac translocates independently of the (p47.sup.phox) and (p67.sup.phox) components, and is essential for oxidase activation. Activation requires dissociation of Rac from rhoGDI so that it can bind GTP. How the cytosolic components interact with the cytoskeleton to cause these translocation events remains unknown. Activation of the oxidase appears to be dependent on lipid mediators, such as phosphatidic and arachidonic acids. Finally, because superoxide anion and its oxidant metabolites are cytotoxic, the NADPH oxidase must be tightly regulated to avoid excessive tissue damage during inflammation. Flavocytochrome-bound Rap 1A, (p40.sup.phox), PR-39, and cationic proteins in azurophilic granules may serve to downregulate the NADPH oxidase.

The NADPH oxidase is relatively inactive under resting conditions. In response to physiologic stimuli or in vitro stimulation with a variety of agents, the preformed subunits of the oxidase rapidly assemble and superoxide is generated at levels ~100-fold greater than at rest. The phagocyte oxidase is activated by ingestion of pathogens or other particulates and by a variety of chemoattractants that bind to pertussis toxin sensitive G-protein coupled receptors. Examples of the latter include N-formyl peptide, C5a, platelet aggregating factor, leukotriene (B.sub.4), and interleukin-8 (24, 401). These chemoattractants mediate signaling through activation of phospholipase C-(Beta), generation of inositol 1,2,5 triphosphate, and release of intracellular calcium, which precedes the respiratory burst (401). Calcium ionophores such as A23187 can also activate the oxidase, as can direct activation of protein kinase C (PKC) through agonists such as PMA, which is not dependent on the release of intracellular calcium.

The pathways that are most physiologically relevant involve phosphorylation. The respiratory burst that results from phagocytosis or chemoattractants is reduced by inhibitors of tyrosine kinase and phosphatidylinositol 3-kinase (PI3-kinase) (25, 273). However, these agents do not suppress the PMA-elicited respiratory burst (98), and PKC inhibitors do not affect the respiratory burst elicited by N-formyl peptide (273). In addition, NADPH oxidase activity elicited by anionic amphophiles such as arachidonic acid and sodium dodecyl sulfate (SDS) do not require protein phosphorylation (279, 311).

The phosphorylation of oxidase components during activation and the ultimate assembly of the functional oxidase are major foci of research. Assembly of the activated oxidase involves translocation of the (p47.sup.phox), (p67.sup.phox), and (p40.sup.phox) to the plasma membrane where they associate with the cytoplasmic side of the flavocytochrome (68, 174, 176, 191,280, 338). (p47.sup.phox), (p67.sup.phox), and (p40.sup.phox) are bound to each other in a large 240-260 kDa complex in the cytosol at rest (191,378). The association of these proteins is mediated in part by SH3 domains forming noncovalent intra- and intermolecular bridges with their target proline-rich motifs (144, 145). During activation, these cytoplasmic components translocate to the plasma membrane, dependent on phosphorylation of (p47.sup.phox) (121, 122, 132, 184, 338).

(p47.sup.phox) is extensively phosphorylated at a group of **C - terminal** serine residues (S303-S379) during activation, acquiring as many as 9 phosphate residues (132, 184). In EBV-transformed lymphocytes from a (p47.sup.phox)-deficient CGD patient, transfection with plasmids containing the (p47.sup.phox) gene with serine (right arrow) alanine mutations in this region resulted in no (p47.sup.phox) phosphorylation, membrane translocation of cytoplasmic components, or oxidase function (132). Single serine (right arrow) alanine substitutions did not impair oxidase function except at position 379, in which translocation and oxidase activity were abolished (132). (p47.sup.phox) S303A and S304A did not support oxidase activity, despite normal translocation of the mutant (p47.sup.phox) (184). Phosphorylation of serine residues 359 or 370 of (p47.sup.phox) is required for subsequent phosphorylation of the remaining serine residues and for translocation (193). At least 1 serine residue of (p47.sup.phox) is phosphorylated after translocation (338), confirming that phosphorylation of (p47.sup.phox) is a coordinated step-wise process, (p67.sup.phox) also undergoes phosphorylation during NADPH oxidase activation, although its physiologic significance is unknown (6, 112, 115, 120, 140, 141).

Src homology domains and other protein-protein interactions: Specific SH3 mediated protein-protein interactions among oxidase components have been identified and their critical physiologic relevance confirmed (90-94, 96, 144, 145, 234, 337, 387) (Figure 5).

(Figure 5 ILLUSTRATION OMITTED)

Binding of anti-(p47.sup.phox) SH3 antibody to native (p47.sup.phox) is dependent on the presence of arachidonic acid, suggesting that in native (p47.sup.phox), the SH3 domains are concealed (387). Arachidonic acid and other anionic amphophiles may induce a conformational change in (p47.sup.phox), unmasking these SH3 domains and allowing for (p47.sup.phox) binding to proline-rich targets on (p67.sup. phox) and (p22.sup.phox) (387). (p47.sup.phox) itself contains a proline-rich segment at its **C - terminus** that may form a hairpin loop folding back, binding to, and masking its own SH3 domains. Phosphorylation at its **C - terminal** serine residue probably leads to a conformational change, exposing masked regions

(300).

The Leto group (91, 92) showed that the SH3 domains in (p47.sup.phox) and (p67.sup.phox) are critical for NADPH oxidase function in intact cells. In cell-free systems, the N-terminal SH3 domain of (p47.sup.phox) binds to the cytoplasmic domain of (p22.sup.phox) and to (p47.sup.phox) itself (92). The **C - terminal** SH3 domain of (p47.sup.phox) binds to the N-terminal region of (p67.sup.phox) (residues 1-154) (92). Induced mutations in either of the (p47.sup.phox) SH3 domains lead to reduction of oxidase function, but only mutations in the **C - terminal** SH3 domain affect (p67.sup.phox) translocation (92). In EBV-transformed lymphocytes from a patient with (p67.sup.phox) CGD, restoration of oxidase activity occurred following transfection with full-length (p67.sup.phox) cDNA, but deletion of either SH3 domain of (p67.sup.phox) caused reduced translocation and superoxide production (91).

C - terminally truncated forms of (p47.sup.phox) and (p67.sup.phox) support oxidase activation without the addition of amphophiles, while full-length protein requires an exogenous amphophile for oxidase activation (167). In transfected cells, the **C - terminally** deleted forms of (p47.sup.phox) and (p67.sup.phox) bind to the membrane without stimulation (90). Thus, the **C - termini** of both (p47.sup.phox) and (p67.sup.phox) may regulate the assembly of the oxidase. Phosphorylation and amphophiles are likely acting by disruption of the intramolecular bond between the N-terminal SH3 (p47.sup.phox) region and the **C - terminus** of the protein, thus exposing the SH3 domain to the flavocytochrome.

The cytoplasmic domains of (p22.sup.phox) and (gp91.sup.phox) are important targets for binding of the cytosolic proteins. Proline to glycine substitution in the proline-rich region of (p22.sup.phox) (position 156) disrupts (p47.sup.phox) binding to (p22.sup.phox) and ablates translocation of the cytosolic subunits to the membrane (236). The **C - terminal** cytoplasmic domain of (gp91.sup.phox) contains the NADPH-binding site and is likely also a docking site for (p47.sup.phox). In vitro, (p47.sup.phox) can bind with both (gp91.sup.phox) and (p22.sup.phox) (94, 96, 234, 387). Recombinant (p47.sup.phox) bound to peptides containing the RGVHFIF motif (559-565 of (gp91.sup.phox)) and peptides derived from this region as well as antibodies raised against this motif inhibited superoxide production (96, 215, 216, 275, 337). However, mutagenesis of residues within RGVHFIF resulted in no or little inhibition of oxidase activity, suggesting that this motif may not be critical for oxidase assembly and function in whole cells (438). The deletion of residues T298-T302 in (gp91.sup.phox) in a (X91.sup.+) CGD patient led to poor translocation of (p67.sup.phox) and (p40.sup.phox) to the membrane, but (p47.sup.phox) translocation was normal (114).

(p40.sup.phox) is a recently described but enigmatic member of the NADPH oxidase complex. (p40.sup.phox) translocates with (p47.sup.phox) and (p67.sup.phox) to the membrane following activation, but is not necessary for oxidase function (93, 113, 114, 345). No case of CGD has been attributed to a defect in (p40.sup.phox). The gene is located on chromosome 22q13.1 and spans ~18 kb with 10 exons. The mRNA is widely distributed in cells that contain the NADPH oxidase as well as in other cell types (T lymphocytes, plasma cells, mast cells, basophils, megakaryocytes, and neuronal cells of the mouse brain), suggesting that its function may not be limited to the NADPH oxidase (265, 436). (p40.sup.phox) copurifies with (p67.sup.phox) and was markedly reduced in a (p67.sup.phox)-deficient CGD patient (and to a lesser degree in (p47.sup.phox)-deficient CGD), suggesting that (p67.sup.phox) may stabilize the (p40.sup.phox) protein (402, 426). There is an interaction between the **C - terminal** domain of (p40.sup.phox) and the inter-SH3 domain of (p67.sup.phox) (113, 274, 345).

(p40.sup.phox) also contains an SH3 domain (residues 175-224) that binds to the **C - terminal** region of (p47.sup.phox) (residues 358-390), and to (p67.sup.phox) (144, 145, 187, 345). Excess recombinant (p40.sup.phox) inhibited oxidase activity in cell-free and cellular systems (34,5). (p40.sup.phox) likely reduces oxidase activity by competing with (p67.sup.phox) SH3-mediated binding to the **C - terminal** proline-rich motif of (p47.sup.phox). Consistent with this model, transfection of K562 cells with the isolated (p40.sup.phox) SH3 domain inhibited whole cell oxidase function more effectively than full-length (p40.sup.phox) (345).

(p40.sup.phox) is phosphorylated by a PKC-type kinase during NADPH oxidase activation at threonine 154, 20 residues upstream of the SH3 domain, and at serine 315, which interacts with the inter-SH3 domain of

(p67.sup.phox) (42). These phosphorylations may induce re-arrangements in the protein-protein interactions between (p40.sup.phox) and the other cytosolic phox proteins necessary for oxidase activation.

Lipid mediators: Phospholipase D (PLD) hydrolyzes the terminal diester bond of membrane-associated glycolipids, forming phosphatidic acid. Different isoforms of PLD are activated by several agents, including chemokines, chemotactic peptides, growth factors, endotoxin, TNF-(Alpha), and PMA (reviewed in reference 157). Several cell-free studies have linked generation of phosphatidic acid with NADPH oxidase activation. Phosphatidic acid and its metabolite, diacylglycerol, synergize in augmenting translocation of (p47.sup.phox) and (p67.sup.phox) to the membrane and in superoxide generation (299, 320).

The mechanism(s) underlying endogenous lipid-mediated NADPH oxidase activation are unknown. Several lipids including phosphatidic acid, arachidonic acid, and phosphatidylinisitols can disrupt the association between Rac and its inhibitor Rho-GDI, allowing uncomplexed Rac to bind to GTP, a necessary step in NADPH oxidase activation (64). Multiple endogenous proteins, including (p47.sup.phox), are phosphorylated following stimulation with phosphatidic acid and diacyl glycerol (260). Phosphatidic acid may activate PKC and induce another protein kinase that phosphorylates (p47.sup.phox) (260, 367, 413). This PA-activated protein kinase has a MW of 125 kDa and is expressed in several cell types (413). NADPH oxidase activation by phosphatidic acid and diacyl glycerol, unlike anionic amphophiles, is dependent on protein kinase function (260).

Dana et al (88) showed that release of arachidonic acid in intact cells is required for oxidase activation. After arachidonic acid is released from membranes by phospholipase (A.sub.2), it can be metabolized to either prostaglandins or leukotrienes.

Endotoxin: Though endotoxin does not directly activate NADPH oxidase, it primes neutrophils to generate higher levels of reactive oxidants in response to other stimuli, such as N-formyl peptide (95). Endotoxin-mediated priming of neutrophils is associated with redistribution of the flavocytochrome from specific granule fractions to plasma membrane-rich fractions and translocation of (p47.sup.phox) to the membrane increased (95). Sequential treatment with endotoxin and N-formyl peptide results in increased translocation of (p47.sup.phox), (p67.sup.phox), and Rac2 and a ~10-fold augmentation of superoxide production compared to either agent alone (95).

Downregulators of NADPH oxidase: PR-39 is a proline-arginine (PR)-rich antibacterial peptide initially isolated from swine neutrophils, and is believed to play a role in wound repair (154, 369). PR-39 likely inhibits NADPH oxidase activity in whole cells and in cell-free assays by binding of its polybasic amino terminal segment to the SH3 domains of (p47.sup.phox) thereby preventing interaction between (p47.sup.phox) and (p22.sup.phox) (369). Recently, it was shown that PR-39 inhibits NADPH oxidase in pulmonary endothelium following ischemia reperfusion injury, suggesting that it may have a role in reducing tissue damage caused by reactive oxidants (7).

Cationic protein constituents of primary azurophilic granules are released into the phagosome after fusion with the cytochrome-containing secondary granules. The human defensin HNP-1, a microbicidal cationic, cystine-rich polypeptide, inhibits NADPH oxidase activity in vitro presumably by interfering with (p47.sup.phox) translocation to the membrane (393). Proteinase 3, a serine protease found in azurophilic granules, also inhibits oxidase activity in stimulated neutrophils by ~50% (394). These findings suggest that azurophil constituents may prevent excessive tissue damage by regulating NADPH oxidase activity (394).

Other potential regulators include the aldehyde 4-hydroxynonenal, a product of lipid peroxidation and nitric oxide (146, 371). Both aldehyde 4-hydroxynonenal and NO are likely to accumulate at inflammatory sites, and thus may be physiologically relevant in regulating the oxidase.

Transcriptional regulation of NADPH oxidase subunits

(gp91.sup.phox), (p47.sup.phox), and (p67.sup.phox) have myeloid-specific expression, whereas (p22.sup.phox) is ubiquitously expressed. However, several nonphagocytic cells such as endothelial cells, fibroblasts, and renal mesangial cells contain NADPH oxidase-like components and can generate low levels of superoxide anion (147, 196, 259, 296, 297). B cells contain all of the components of the phagocyte NADPH oxidase, and generate superoxide upon stimulation with various agonists,

but at a far lower level than neutrophils, perhaps due to lower levels of (sup.phox) proteins (114).

Several transcription factors are involved in regulation of (gp91.sup.phox) (see Table 1). CP1, a ubiquitous transcriptional factor which binds to the CCAAT box motif, binds with the (gp91.sup.phox) promotor (374). BID (binding increased during differentiation) also binds to the (gp91.sup.phox) promotor at sites overlapping with CP1 (248). CDP (CCAAT displacement protein) may repress transcription of the (gp91.sup.phox) gene by competing with CP1 and BID (248, 374). Deletion of CDP binding sites leads to increased (gp91.sup.phox) promotor activity (248). CDP binding to (gp91.sup.phox) occurs at 4 separate sites, and binding to each site is reduced during terminal phagocytic differentiation in association with (gp91.sup.phox) expression. Therefore, the restricted expression of (gp91.sup.phox) to myeloid cells may be controlled by competition for multiple promotor binding sites by transcriptional activators and repressors (248).

In 2 separate kindreds with X-CGD, single base pair mutations were identified at positions -55 and -57 in the (gp91.sup.phox) promotor, respectively (119). These mutations lead to impaired binding of a protein complex, termed hematopoiesis-associated factor 1 (HAF1), to the (gp91.sup.phox) promotor (119). HAF-1 is composed of 3 transcription factors: PU.1, interferon regulatory factor 1 (IRF-1) and interferon consensus sequence binding protein (ICSBP) (117). Each of the 3 transcriptional factors bind to the (gp91.sup.phox) promotor, and cotransfection of all 3 transcription factors leads to a marked increase in promotor activity of the (gp91.sup.phox) gene in myeloid cell lines (117, 368). HAF-1 is also necessary for interferon-(Gamma)-(IFN-(Gamma)) induced upregulation of (gp91.sup.phox) expression (119).

PU.1 is an ets transcriptional factor that is critical for terminal neutrophil maturation (14). In in vitro assays, PU.1 increases promotor activity of several myeloid specific genes, including (Beta)2 integrins (CD11b/CD18), neutrophil elastase, and the Fc(Gamma) receptors I and III as well as receptors for macrophage-, granulocyte-and granulocyte-macrophage-colony-stimulating factor (133, 179, 290, 298, 310, 333, 375, 437). Neutrophils from PU.1 null mice lack (gp91.sup.phox) mRNA and protein, and consequently do not generate superoxide anion (13). PU.1-deficient neutrophils also fail to respond to chemokine signals, and have defective phagocytosis and intracellular killing (14). PU.1-deficient mice die within 2 days of birth from systemic bacterial infection (13).

Eklund et al (117) and Suzuki et al (390) showed that X-CGD patients with mutations at -55 and -57 or -52 and -53 in the (gp91.sup.phox) promotor had defective binding of PU. In 1 X-CGD patient, eosinophils expressed normal quantities of (gp91.sup.phox) and generated superoxide anion (225), indicating that (gp91.sup.phox) expression in eosinophils is regulated differently than in neutrophils, monocytes, and B lymphocytes, and likely does not require PU.1 (390). Recently, GATA-3, an eosinophil-specific transcription factor, was shown to regulate negatively (gp91.sup.phox expression in eosinophil-committed HL-60-C15 cells (342).

PU.1 is also essential for (p47.sup.phox) promotor activity, with a binding site between -37 and -52 (243). The (p47.sup.phox) promotor is active in myeloid but not in nonmyeloid cell lines, likely due to differential PU.1 expression or binding. Directed mutations abolished PU.1 binding and lead to loss of promotor activity (243). Cotransfection of nonmyeloid cells with PU.1 and a (p47.sup.phox) gene promotor-luciferase construct led to induction of promotor activity. These findings contrast with those in (gp91.sup.phox) in which lineage-specific expression requires both PU. 1 and the IRF transcription factors (117).

NADPH oxidase activity is augmented in vitro by a variety of cytokines (for example, IFN-(Gamma); TNF-(Alpha); granulocyte-, macrophage-, and granulocyte-macrophage-colony-stimulating factors), and is downregulated by dexamethasone (72, 95, 283). In THP-1 cells, a myeloid cell line, IFN-(Gamma) and TNF-(Alpha) individually and synergistically increase superoxide production, cytochrome (b.sub.558) content and expression of (gp91.sup.phox) and (p47.sup.phox) genes, whereas dexamethasone decreases oxidase activity and expression of these genes (72).

Diagnosis of CGD

Early laboratory methods used to support the, diagnosis of CGD included measurements of oxygen consumption and bactericidal studies

involving patient neutrophils. Baehner and Nathan (23) noted that CGD leukocytes failed to reduce NBT dye to a blue formozan precipitate during phagocytosis. The NBT test is also useful in identifying carrier female relatives of male CGD patients in whom peripheral leukocytes consist of 2 cell populations, only I of which reduces NBT (429). The proportion of NBT-positive leukocytes varies among individual female carriers, consistent with random inactivation of the X chromosome in female carriers (429). None of the female carriers identified by Baehner and Nathan had clinical CGD, indicating that a threshold proportion of normal leukocytes is adequate for normal host defense. The level of this threshold is important in management strategies, as discussed below.

The NBT test is based on microscopic evaluation of a limited number of cells. Rather than detecting a "respiratory burst" per se, the assay relies on the accumulation of formozan precipitate over time. Thus in some cases of (A.sup.+) (autosomal recessive, cytochrome positive) or (X.sup.+) CGD in which low levels of oxidants are produced, the NBT test may accumulate positive staining over time. We and others have encountered patients with apparently normal NBT tests in whom impaired oxidant function was subsequently documented by more discriminant tests (244, 410). Thus, patients with a compatible clinical history of CGD and a qualitatively normal NBT test should be evaluated by a quantitative measurement of phagocyte oxidase function.

Quantitative methods for evaluating oxidase activity include the measurement of superoxide production by ferricytochrome c reduction and the use of probes whose chemiluminescent or fluorescent properties are altered by their reaction with reactive oxidants (Table 3). The diagnostic method currently employed in our lab is a fluorescent assay using the conversion of dihydroxyrhodamine 123 (DHR) to rhodamine 123, which detects hydrogen peroxide (410-412) (Figure 6). The DHR method can not only diagnose CGD but also suggest the CGD genotype. Stimulated neutrophils from patients with (p47.sup.phox)-deficient or (X.sup.+) (cytochrome positive) CGD generate a small but measurable level of hydrogen peroxide by DHR as distinct from the more common (X.sup.0) CGD phagocytes, which do not (75, 124, 411). This residual oxidant production may account for the better prognosis in the autosomal recessive and (X.sup.+) forms of CGD (124, 244, 411).

(Figure 6 ILLUSTRATION OMITTED)

TABLE 3. Diagnostic methods in CGD

Nitroblue tetrazolium reduction (NBT)
Ferricytochrome c reduction
Chemiluminescence
Dihydrorhodamine and other fluorescence assays
(H.sub.2)(O.sub.2) production (scopoletin oxidation)

Once the diagnosis of CGD is made the genotype can be determined. A mosaic population of oxidase-positive and -negative neutrophils in a male patient's mother or sisters strongly suggests X-linked CGD. Lack of a mosaic pattern among female relatives does not rule out the X-linked mode of inheritance because the defect can arise spontaneously. Absence of the membrane-bound flavocytochrome subunit by spectral or immunoblot analysis indicates a defect in either the (gp91.sup.phox) or (p22.sup.phox). Definitive methods for establishing the CGD genotype include immunoblotting for (p47.sup.phox) and (p67.sup.phox), or direct sequencing of the (gp91.sup.phox) and (p22.sup.phox) genes, since absence of either cytochrome protein generally leads to loss of the other.

Prenatal diagnosis of CGD can be done by analysis of neonatal neutrophil oxidant production from umbilical vein samples obtained by fetoscopy. This can be performed well only into the second trimester, at substantial fetal risk (282). Oxidant production from amniocytes is unreliable (359). Alternatively, DNA can be analyzed from amniocytes or chorionic villus samples (331). Intrageneic RFLPs have been identified in the (gp91.sup.phox) (30, 271, 307) and in the (p67.sup.phox) gene (207). Recently, Gorlin (160) identified highly polymorphic (CA/GT)n repeats at 2 locations within the (gp91.sup.phox) gene that are promising tools for prenatal diagnosis. When specific mutations within a family are known, direct sequencing of fetal DNA should be performed.

Mutations in CGD

Defects in the (gp91.sup.phox) gene account for approximately two-thirds of CGD cases, and mutations in the (p47.sup.phox) gene account for most of the remaining cases. Mutations may lead to total absence of protein, decreased amount of protein, or a defective protein product expressed at normal levels.

Rae et al (324) identified mutations in the CYBB gene in 131 consecutive X-linked CGD kindreds. Single-strand conformational polymorphism (SSCP) analysis identified defects in 124 of the patients; mutations in the remaining 7 kindreds were identified by sequencing. The 103 specific mutations identified throughout the 13 exons of the gene included deletions (11%), frameshifts (24%), nonsense mutations (23%), missense mutations (23%), splice-region mutations (17%) and regulatory-region mutations (2%). No single mutation occurred in more than 7 kindreds. The most common mutation was a frameshift due to an insertion of an adenosine nucleotide at position 749 where 6 consecutive adenosines may predispose to recombination errors. Of 87 mothers evaluated, 77 (89%) were X-linked CGD carriers, indicating that de novo mutations occur in ~10% of cases. In 7 unrelated South American families with X-linked CGD, a high degree of molecular heterogeneity was also observed (305). Therefore, X-linked CGD results from a heterogenous spectrum of mutations in the (gp91.sup.phox) gene, without an apparent founder effect (305, 324).

Francke et al (143) showed that X-linked CGD mutations arose de novo from unaffected maternal grandfathers in 2 families. Ariga et al (17) found a significant rate of sporadic carriers arising from sperm in 13 families with X-linked CGD. A larger data base is required to confirm whether an imbalance in the mutation rate in male and female gametes exists. An imbalance in sexual mutation rate has been reported in the X-linked disorders Duchenne muscular dystrophy (OMIM 310200) (26) and hemophilia A (OMIM 3067000) (33).

In most cases of X-linked CGD, (gp91.sup.phox) is absent ((X91.sup.0)). An X-linked variant form of CGD exists in which the flavocytochrome content is 1%-25% of normal ((X91.sup.-)), but constitutes (is less than) 10% of cases (40, 239, 284, 330, 361). (X91.sup.-) phagocytes are capable of generating reduced but measurable amounts of superoxide, and NBT testing generally shows a homogenous population of weakly staining cells. (X91.sup.-) cases arise from missense mutations or small in-frame deletions. Large deletions, nonsense mutations, and most splice-junction mutations lead to X91(0) CGD. Some (X91.sup.-) cases are associated with a reduced affinity for NADPH (239, 284, 361). The X-CGD mutation His-338 to Tyr occurs at the predicted FAD binding motif, and causes diminished expression of (gp91.sup.phox) and abolishes FAD binding (433). Most patients with X91- CGD are diagnosed later in life than (X91.sup.0) patients, and have a milder clinical course (361,386).

In the few cases of (X91.sup.+) CGD reported, normal amounts of the cytochrome are present in neutrophil membranes, but the cytochrome is nonfunctional. In 2 unrelated kindreds, a Pro415 to His caused impaired NADPH and FAD binding (78, 102, 324). Arg54 to Ser caused a defect in the heme center of the NADPH oxidase (78). Asp500 to Gly led to defective translocation of (p47.sup.phox) and (p67.sup.phox) to the membrane (237).

Roesler et al (328) reported a German series in which 6 of 9 patients with X-linked CGD had the (X91.sup.+) or (X91.sup.-) forms. In only 3 cases of variant X-CGD did cytochrome levels correlate with oxidase activity. The variant patients without correlation of cytochrome levels and oxidase activity consisted of 3 missense mutations, 1 affecting the putative NADPH binding site (Leucine 546 (right arrow) Proline) and 2 affecting the putatative FAD binding site (Pro 339 (right arrow) His and His 338 (right arrow) Tyr).

Defects in (p22.sup.phox) account for ~5% of CGD cases. In most cases of (p22.sup.phox)-deficient CGD, no flavocytochrome is present ((A.sup.-) CGD). An exception involved a proline to glutamine substitution in a proline-rich region of (p22.sup.phox) involved in binding to the SH3 domain of (p47.sup.phox) (105, 236). In 9 characterized families with (p22.sup.phox) CGD, 10 different mutations were found in 18 alleles studied (263, 331).

In contrast to the other forms of CGD, the vast majority of (p47.sup.phox) CGD is due to the same mutation (159). In studies of 10 patients with (p47.sup.phox) CGD from the United States, Europe, and Japan, 19 of 20 alleles contained a GT deletion at the beginning of exon 2

predicting a flame shift and a premature stop codon at amino acid 51 (59, 159, 189, 406). A closely linked (p47.sup.phox) pseudogene that carries the GT deletion was found in genomic DNA from 34 consecutive unrelated normal subjects (159). The wildtype and pseudogene structures were similar, both containing 11 exons and 10 introns. In addition to the GT deletion, the pseudogene had a 30-bp deletion in intron 1 and a 20-bp duplication in intron 2, and a number of single base changes. The authors hypothesize that aberrant recombination events between the wildtype and pseudogene account for the high prevalence of GT-deletants in the (p47.sup.phox-)/- form of CGD (159).

Defects in (p67.sup.phox) account for ~5% of CGD cases. Of 11 patients studied, 12 different mutations have been found (263, 331). The (p67.sup.phox) protein was absent in all but 1 reported case in which a deletion of a lysine residue at position 58 was associated with diminished binding to Rac1 (238).

X-Linked CGD Carriers

Early in embryogenesis, 1 of the 2 X chromosomes in all somatic cells of females becomes inactivated (lyonization) (249). Based on the binomial distribution of the proportion of normal neutrophils in X-linked CGD carriers, it was estimated that lyonization occurred during embryogenesis at a stage when only 8 hematopoietic founder cells were present (48). Once X-inactivation occurs in a given cell, it is believed that the same X chromosome is inactivated in all progeny. In some X-linked diseases such as SCID (OMIM 308380) (55) or Wiskott-Aldrich syndrome (OMIM 301000) (304), proliferation of hematopoietic cells expressing the nonmutated X chromosome is favored. In contrast, in X-linked CGD carriers, there is no apparent growth advantage for oxidase-competent or -incompetent cells (48).

The most common phenotypes in X-CGD carriers are cutaneous lesions resembling discoid lupus clinically and histologically, and recurrent aphthous stomatitis (43, 137, 155, 162, 245, 247, 431). Such skin lesions have been observed in patients with X-linked and autosomal recessive CGD as well (28, 372, 384, 385). Although most carriers with ~10% normal phagocytes have normal host defense, in rare instances, carriers with the same or a greater proportion of normal circulating granulocytes may have clinical evidence of a host defense defect.

Johnston et al (195) reported an X-CGD carrier with recurrent bacterial infections suggestive of CGD with a variable fraction of normal circulating granulocytes ranging from 4% to 44% over 4 years. Kaplan et al (204) reported an (X.sup.0)CGD carrier in good health until age 66 years when a granulomatous lung lesion developed. At age 68 years, she died of Burkholderia cepacia and Trichosporon beigelii sepsis. A preterminal NBT test showed 9% of neutrophils were reactive, whereas previously, 20% of neutrophils had been. Therefore, in X-CGD carriers, the degree of mosaicism may vary over time. Other genetic factors, such as nonoxidative antimicrobial pathways, may influence the overall clinical phenotype, as well.

X-linked female carriers with skewing of neutrophil populations toward oxidase-defective severe enough to cause the clinical phenotype of CGD have been reported. Curnutte et al (83) reported a female heterozygote whose neutrophils uniformly failed to generate superoxide. We recently identified a (gp91.sup.phox+/-) female with phenotypic CGD and only 0.3% oxidant-producing circulating granulocytes by the DHR assay (unpublished observations). In such patients, the genotype should be established molecularly given the risk to male offspring. There are reports of phenotypic disease occurring in female carriers of Wiskott-Aldrich syndrome, Hunter syndrome (OMIM 309900), hemophilia, and Lesch-Nyhan syndrome (OMIM 308000), due to heavily skewed X-chromosome inactivation (16, 69, 286, 304).

The XIST gene has been identified as the regulatory switch expressed on the X chromosome that becomes inactivated (OMIM 314670) (171, 309). A mutation in the promotor of the XIST has been reported in 2 unrelated families with preferential inactivation of the X chromosome carrying the mutation (314). Therefore, in some X-linked carriers with an unusually high proportion of mutant cells, X-chromosome inactivation may be skewed by an alteration in expression of 1 or more X-linked genes or conceivably by trans-activating autosomal genes that regulate inactivation.

Pathogens in CGD

CGD patients have recurrent infections with a subset of catalase-producing pathogens (152, 229, 268, 396). Kaplan et al (203)

originally showed that the bactericidal defect in CGD was not global, but rather quite specific. In vitro killing of S. aureus and Serratia marcescens by CGD neutrophils was markedly impaired, while bactericidal activity against streptococci was entirely normal.

Klebanoff et al (210, 212, 214) and McRipley and Sbara (262) noted that bactericidal activity of phagocyte homogenates was markedly increased when (H.sub.2)(O.sub.2) and a halide (iodide or chloride) were added. Leukocyte myeloperoxidase can convert (H.sub.2)(O.sub.2) to hypohalous acid (see Figure 3)), normally in CGD, but (H.sub.2)(O.sub.2) is not made by CGD neutrophils due to the defect in the NADPH oxidase. Streptococci do not degrade their own (H.sub.2)(O.sub.2) with catalase and therefore provide (H.sub.2)(O.sub.2) which interacts with the myeloperoxidase and halide within phagocytic vacuoles resulting in the formation of hypohalous acid. In CGD phagocytes, catalase-negative (H.sub.2)(O.sub.2)-producing bacteria bypass the defect in CGD and provide bactericidal reagents. Consistent with this hypothesis, a pneumococcal mutant that produced little (H.sub.2((O.sub.2) was not killed by CGD granulocytes (313). The corollary is that in CGD, catalase-producing pathogens such as S. aureus and various Gram-negative bacilli may scavenge their own (H.sub.2)(O.sub.2), and evade reactive oxidant-mediated defense mechanisms.

TABLE 4. Infections in CGD: Common pathogens and sites of involvement

Pathogen	Presentation
Bacterial	
Staphylococcus aureus	Soft tissue infection, lymphadenitis, liver abscess, osteomyelitis, pneumonia, sepsis
Burkholderia (Pseudomonas) species B. cepacia B. gladioli B. pseudomallei	Pneumonia, sepsis
Serratia marcescens	Pneumonia, osteomyelitis, sepsis, soft tissue infection
Nocardia species N. asteroides N. nova N. otitidiscaviarum N. farcinica	Pneumonia, osteomyelitis, brain abscess
Chromobacterium violaceum	Soft tissue infection, sepsis
Fungal	
Aspergillus species A. fumigatus A. nidulans A. flavus A. terreus A. niger	Pneumonia, osteomyelitis, brain abscess
Paecilomyces species P. variotti P. lilacinus	Pneumonia, soft tissue infection, osteomyelitis
Phaeohyphomycete species (dark-walled fungi) Exophiala sp. Bipolaris sp. Cladosporium sp.	Pneumonia, soft tissue infection
Penicillium species	Pneumonia, soft tissue infection
Miscellaneous filamentous	

fungi and yeasts	
Zygomycete species	
Acremonium sp.	
Trichosporon beigeli	
Trichosporon inkin	
Candida species C. albicans Torulopsis (Candida) glabrata	Sepsis, soft tissue infection, liver abscess

A registry of United States residents with CGD was created in 1992 to characterize the epidemiology and clinical features of this disease (429a). The database is composed of 368 patients (259 X-linked, 81 autosomal recessive, 28 unknown). Table 4 summarizes the most common sites of infections and the most common pathogens at each site. Other pathogens that afflict CGD patients include molds other than Aspergillus species (for example, Paecilomyces species, dark-walled molds) (205, 206, 373, 428), Salmonella species (268), Nocardia species (1, 47, 58, 135, 194, 198), Chromobacterium violaceum (251, 379), and possibly tuberculous (125, 228) and nontuberculous (11, 65, 291) mycobacteria. CGD patients are not at increased risk for catalase-negative pathogens, such as streptococci.

The paradigm of catalase-dependent virulence in CGD is incomplete, since only a subset of catalase-positive pathogens is encountered in CGD. Pseudomonas aeruginosa is a catalase-positive pathogen that is rarely encountered in CGD, whereas Burkholderia (Pseudomonas) cepacia and less commonly other Burkholderia species are important CGD pathogens that are only rarely encountered in other immunocompromised patients (107, 334, 380). Consistent with the clinical experience, Speert et al (380) showed that neutrophils from CGD patients had normal killing of P. aeruginosa, while killing of B. cepacia was markedly impaired.

Chromobacterium violaceum, a rare pathogen typically encountered in brackish waters, occurs with unusually high frequency in CGD patients from the southern United States (for example, Florida, Louisiana), although C. violaceum infection in the northern United States has been reported (252, 379). Clinical manifestations include soft tissue infection, pneumonia, and sepsis.

Aspergillus is 1 of the most important causes of morbidity and mortality in CGD (70, 268, 269). A. fumigatus was the most common species isolated in CGD patients at the National Institutes of Health (NIH) (Bethesda, MD), but A. nidulans was more virulent based on the mortality, propensity to spread from the lung to adjacent structures, and dissemination (357). Invasive A. nidulans is rarely observed in other immunocompromised patients at high risk for aspergillosis (for example, patients with neutropenia or bone marrow transplant recipients), but several reports exist of A. nidulans infection in CGD (50, 60, 70, 208, 281, 294, 357, 381, 403, 424).

Mouse knockout models of CGD have shed light on pathogen virulence factors (192, 315). Catalase-deletant strains of A. nidulans were as virulent as wildtype A. nidulans in CGD mice, whereas wildtype mice had no morbidity (61). Future studies with bacterial pathogens rendered catalase deficient should help clarify the role of bacterial catalase in CGD infections.

In vivo, there is evidence that the reactive oxidant and reactive nitrogen pathways have discrete effects on host defense and inflammation, and in some settings, interact with 1 another. In a cobra venom toxin model of lung injury, X-CGD mice, but not wildtype mice, are protected by pretreatment with an iNOS inhibitor, suggesting that either NADPH oxidase or iNOS can mediate lung injury, but simultaneous disruption of both pathways abrogates injury (224). Shiloh et al (370) intercrossed X-CGD mice with transgenic iNOS-deficient mice, and showed that double-knockout mice were far more immunocompromised than either of the parental strains. The double-knockouts developed fatal spontaneous infections with indigenous enteric flora. In vitro bactericidal activity of macrophages from double knockouts against a variety of bacterial pathogens was reduced but was not nil, suggesting that host defense pathways independent of NADPH oxidase and

iNOS exist (370).

Inflammatory Complications

In addition to recurrent infections, CGD patients frequently have exuberant and persistent tissue granuloma formation. The clinical manifestations of chronic inflammation are varied and include skin ulceration, excessive inflammation at drainage and surgical wounds leading to dehiscence, autoimmune diseases resembling systemic lupus erythematosus, discoid lupus, pneumonitis, and inflammatory bowel disease resembling Crohn disease clinically and histologically (28, 29, 152, 230, 256, 293, 349, 423). Granulomatous involvement of hollow viscera may lead to gastrointestinal and genitourinary obstruction (9, 89, 404, 416) (see Figure 2). These inflammatory complications typically respond rapidly to systemic steroid therapy (63, 89). Cyclosporine and sulfasalazine have been used for refractory small and large bowel (disease (29, 332).

The mechanisms underlying abnormal inflammatory responses in CGD are undefined. In some cases, granulomatous complications arise from unresolved infection. However, inflammatory sites are frequently sterile, and evidence exists in both CGD patients and mouse models of CGD that exuberant inflammation occurs independent of infection. Gallin and Buescher (151) showed that X-linked CGD patients have increased neutrophilic exudate compared to normal volunteers in a skin window model. In both the X-linked (315) and (p47.sup.phox-/-) (192) mouse models of CGD, increased peritoneal neutrophilic leukocytosis develop compared to wildtype mice following intraperitoneal challenge with the sterile irritant thioglycollate. In X-linked CGD mice, abnormally exuberant acute and chronic inflammatory responses occur following intratracheal challenge with heat-killed A. fumigatus hyphae (266). We have recently shown that (p47.sup.phox-/-) mice generate normal granuloma formation and T-cell mediated cytokine responses to challenge with Mycobacterium avium and Schistosoma mansoni eggs, suggesting that exuberant inflammatory responses in CGD likely do not arise from an abnormality in the T-cell phenotype (358). Appropriate regression of established tissue granulomata in (p47.sup.phox-/-) mice challenged with Schistosoma mansoni eggs suggests that persistent granulomata in CGD may be stimulus-dependent as opposed to an invariant feature of the disease (358).

One mechanism that may relate to increased inflammation in CGD is the inability of CGD neutrophils to inactivate the proinflammatory chemoattractants, leukotriene B4, leukotriene C4, C5a, and N-formyl peptide. Catabolism of these inflammatory mediators in vitro by neutrophils and in cell-free systems is dependent on reactive oxidant generation (66, 164, 170).

Foster et al (142) evaluated polymorphisms in 7 candidate host defense genes in CGD patients with and without a history of inflammatory complications. In each candidate gene, previous studies suggested that the polymorphism might affect expression or function of the protein product. Polymorphisms of the genes for myeloperoxidase and Fc(Gamma) receptors were strongly associated with gastrointestinal complications, whereas rheumatologic complications were associated with variants of mannose binding lectin and Fc(Gamma)RIIa (142). Thus, polymorphisms in genes unrelated to the NADPH oxidase may modify the clinical phenotype in CGD (142).

Management

CGD patients often do not have typical symptoms and signs of infection (152). Even in the setting of life-threatening infections, CGD patients may be asymptomatic or have mild nonspecific symptoms. Fever and leukocytosis may be absent, and an elevated sedimentation rate may be the only abnormal laboratory test (152). In a review of aspergillosis in CGD patients at the NIH, one-third of patients were asymptomatic at diagnosis and only ~20% were febrile (357). In many of these patients, a pulmonary infiltrate on routine screening chest X-ray or computed tomography (CT) scan was the first indication of an infection. The white blood cell count was (is less than or equal to) 10,000/(micro)L in 13/23 cases and the sedimentation rate was (is less than or equal to)40 mm/hr in 9/20 cases. Therefore, a high level of vigilance in searching for infection is necessary in caring for CGD patients. Frequent radiographic evaluation (for example, chest radiographs during routine clinic visits and CT scans in patients with fever or focal signs) are critical to making early diagnoses.

Although many different organisms have been reported in CGD, the overwhelming majority are caused by only 5 pathogens: S. aureus, B. cepacia, Serratia marcescens, Nocardia spp., and Aspergillus spp. Since the

optimal antimicrobial therapy for these organisms is different and the possibility of rare organisms is high, a microbiologic diagnosis should be pursued vigorously in every instance before initiation of antimicrobial therapy. We find a much higher diagnostic yield from fine-needle aspiration of pneumonias than bronchoscopy. For liver abscesses, which are almost always caused by S. aureus, we find surgery to be necessary in the vast majority of cases.

Several retrospective studies have shown that prophylaxis with trimethoprim-sulfamethoxazole reduces the rate of serious infections in CGD patients (152, 258, 268, 420). Prophylaxis with trimethoprimsulfamethoxazole is effective in X-linked and auto-somal recessive CGD, and reduced the rate of infections by at least 50% in each series with activity against most of the bacterial pathogens commonly encountered in CGD (for example, S. aureus, Serratia marcescens, and Burkholderia and Nocardia species). It is generally well tolerated, and widespread use of trimethoprim-sulfamethoxazole has not led to either increased rates of fungal infections or increased frequency of pathogens resistant to trimethoprim-sulfamethoxazole in our CGD patients (258). For patients with allergic reactions, prophylaxis with trimethoprim alone or dicloxacillin may be considered.

In a European prospective open-label study of itraconazole prophylaxis, the rate of Aspergillus infections was reduced compared with historical controls, and the drug was well tolerated (269). A randomized double-blinded study of itraconazole prophylaxis is currently being performed at the NIH.

Interferon-(Gamma)

Interferon-(Gamma) is a macrophage-activating factor that is critical in host defense against intracellular infections such as Leishmania and Mycobacteria species in humans and mouse models (20, 73, 108, 139, 180, 181,199, 272, 278, 285). IFN-(Gamma) administered in vivo or in vitro augments reactive oxidant generation in mouse peritoneal macrophages (272). In clinical trials in patients with cancer (276) and with lepromatous leprosy (277), IFN-(Gamma) increased (H.sub.2)(O.sub.2) generation in circulating monocytes, and provided a rationale for evaluating IFN-(Gamma) in CGD.

Addition of recombinant IFN-(Gamma) in vitro to phagocytes from some, but not all, CGD patients augmented superoxide production (128, 350). Augmentation of superoxide production was observed in phagocytes from (X.sup.+) CGD and the majority of autosomal recessive CGD patients, as well as in ~30% of (X.sup.0)CGD patients. In a study of patients with (X.sup.+) CGD, 2 consecutive subcutaneous injections of IFN-(Gamma) resulted in a 5- to 10-fold increase in superoxide production from granulocytes and monocytes (127). The effect lasted for more than 2 weeks and was associated with an increase in bactericidal activity. In addition, levels of cytochrome b expression increased from near 0 to 10%-50% of normal values (127).

Given these promising results, a multicenter, randomized, double-blinded, placebo-controlled study of prophylactic IFN-(Gamma) (50 (micro)g/(m.sup.2) subcutaneously thrice weekly) was conducted (185). One hundred twenty-eight CGD patients (67% X-linked and 33% autosomal recessive) were enrolled. Prophylactic IFN-(Gamma) reduced the number of serious infections by over 70%, and was beneficial in both the X-linked and autosomal recessive forms of CGD. In contrast to earlier studies, no significant differences occurred between the IFN-(Gamma) and placebo groups with regard to reactive oxidant generation, cytochrome b expression, or in vitro bacterial killing. Subsequent studies in CGD patients confirmed that IFN-(Gamma) did not improve NADPH oxidase function or increase levels of its constituent proteins (270, 430).

The benefit of IFN-(Gamma) prophylaxis in CGD likely occurs through augmentation of oxidant-independent antimicrobial pathways. IFN-(Gamma) increases TNF-(Alpha) production, tryptophan metabolism, granule protein synthesis, and MHC-II expression (reviewed in reference 153). Recently, IFN-(Gamma) administered to normal volunteers was shown to increase expression of Fc(Gamma)R1 receptors on phagocytes leading to enhanced phagocytosis, and to increased ((Beta).sub.2)-integrin expression on monocytes, which the authors suggest may improve phagocyte trafficking in vivo (347).

The optimal dose, frequency, and route (for example, subcutaneous, aerosolized) of administration remain to be determined. Ahlin et al (4)

reported that in a small group of patients with X-linked and autosomal CGD, administration of IFN-(Gamma) at 100 (micro)g/(m.sup.2) led to enhancement of ex vivo neutrophil functions compared with CGD patients receiving the standard 50 (micro)g/(m.sup.2) dose. The increase in expression of Fc(Gamma)R1 on neutrophils and ex-vivo killing of A. fumigatus hyphae was greater in the high-dose (100 (micro)g/(m.sup.2)) versus standard-dose (50 (micro)g/(m.sup.2)) groups; superoxide generation was augmented in some patients, but did not correlate with the dose of IFN-(Gamma).

Our 7-year follow-up phase IV study of CGD patients receiving IFN-(Gamma) (50 (micro)g/(m.sup.2)) suggests that the drug is, in general, well tolerated and the benefits are likely sustained over and above that achieved with antibacterial prophylaxis alone. Further studies aimed at comparing the prophylactic benefit of different IFN-(Gamma) regimens are warranted. In addition, we do not know whether the therapeutic use of IFN-(Gamma) after the onset of infection is beneficial.

In (p47.sup.phox-/-) mice, prophylactic subcutaneous IFN-(Gamma), reduced the rate of spontaneous infections. Further studies using knockout mice will be helpful in shedding light on the mechanisms associated with IFN-(Gamma) in CGD.

Granulocyte transfusions

Granulocyte transfusions in CGD are supported by the principle that a small number of normal phagocytes may be able to compensate for the oxidative defect in CGD phagocytes. X-linked CGD carrier females have a mosaic population of both normal and CGD. Phenotypically normal carrier females have been identified with as low as 3%-5% circulating neutrophils (48, 253, 254), suggesting that a small proportion of normal granulocytes are sufficient to prevent life-threatening infections.

In vitro, a small proportion of normal neutrophils mixed with CGD neutrophils kill A. fumigatus hyphae as well as normal neutrophils (325). The likely explanation is that (H.sub.2)(O.sub.2) generated by normal neutrophils can diffuse into CGD neutrophils and provide the necessary reagent to generate hypohalous acid and hydroxyl anion (292, 325) (see Figure 3).

Transfused granulocytes have respiratory burst activity and appear to traffic normally based on their recovery from sites of infection (for example, wound drainage sites from liver abscesses), from mouth rinse preparations, from bronchoalveolar lavage, and from neutrophil exudate in an experimental skin window (49, 123, 151).

Several case reports exist of use of granulocyte transfusions to treat serious bacterial and fungal infections in CGD (49, 97, 111, 123, 130, 152, 294, 409, 432). However, the value of granulocyte transfusions in CGD has not been evaluated in a prospective controlled trial. Over the past 20 years, we have used granulocyte transfusions in CGD in life-threatening infections and in infections refractory to antimicrobial and surgical treatment. Granulocyte transfusions are generally well tolerated, but adverse effects include fevers, development of leukoagglutinins leading to rapid loss of transfused granulocytes, and rarely, pulmonary leukostasis. The likelihood of pulmonary leukostasis may be increased if amphotericin B and granulocytes are administered concomitantly; therefore we space granulocyte transfusions and amphotericin B several hours apart. In cases in which systemically administered granulocytes are rapidly consumed due to leukoagglutinins, local instillation of normal granulocytes into the infected site may be beneficial (231). One concern regarding granulocyte transfusions is alloimmunization, which may be of concern for patients under consideration for bone marrow transplantation.

Bone marrow transplantation

Because CGD results from a defect in hematopoietic stem (cells, bone marrow transplantation (BMT) is a rational option to establish a stable population of normal myeloid progenitors. BMT has been reported in at least 10 CGD patients and was curative in all 6 reported cases since 1984 (54, 178, 200, 201, 360). Most cured patients had 100% circulating donor myeloid cells. In 1 patient, a stable chimeric population of circulating granulocytes consisting of 10%-15% normal cells conferred normal protection against infections, similar to X-CGD carriers (200, 201, 360).

The morbidity and mortality (~10%) associated with BMT have mitigated against its routine use in CGD. BMT may be most useful in patients who have had recurrent serious infections despite antibiotic and IFN-(Gamma) prophylaxis and who have HLA-matched normal siblings. Ideally, infections should be under control before initiating BMT. However, the Seger group

(294) has reported a curative transplant in a patient with X-CGD and disseminated A. nidulans infection.

CGD is an attractive candidate disease for stem cell transplantation employing a relatively nonmyeloablative conditioning regimen as an alternative to conventional cytoreductive conditioning. Theoretically, a transplant that led to a stable mixed chimeric myeloid population consisting of as low as 5% donor cells could be curative in CGD, and should have reduced morbidity compared to conventional transplantation.

Gene therapy

CGD is an ideal candidate disease for hematopoietic stem cell gene therapy (253, 254). Engraftment of a stable population of normal myeloid stem cells is curative in CGD, as illustrated by successful bone marrow transplantation (360). Transfer of the relevant gene corrected the NADPH oxidase defect in EBV-transformed B cell lines and in primary monocytes from CGD patients, confirming that expression of the missing protein was sufficient to restore oxidase function in intact cells (62, 106, 255, 317, 318, 399, 400, 406). Reconstitution of phagocyte NADPH oxidase activity has been achieved in the X-linked and autosomal recessive forms of CGD by transduction of patient bone marrow and peripheral blood stem cell progenitors with retroviral vectors containing the relevant gene (106, 240, 316, 363, 421). In the X-linked and (p47.sup.phox-/-) mouse models of CGD, genetic correction of NADPH oxidase has been achieved in vivo with stem cell gene therapy, and gene-corrected mice have increased resistance to experimental infection (35, 257). Based on the experience in X-linked CGD carriers, only a small proportion of normal phagocytes are required for normal host defense.

The goal of gene therapy in CGD is to achieve a stable population of gene-corrected myeloid precursors that would generate a biologically significant number of peripheral phagocytes. In the short term, it is conceivable that infusions of gene-corrected progenitors that persist for a few months may be beneficial as adjunctive therapy for serious infections (254). Successful gene therapy in CGD and in other hematopoietic disorders is limited by the transduction efficiency of myeloid precursors ex vivo and by the ability of corrected cells to replace uncorrected cells in vivo. In certain hematopoietic diseases such as X-linked SCID, gene-corrected cells may have a selective growth advantage both ex vivo and in vivo (55, 232, 288). No such selective growth advantage is thought to exist in gene-corrected cells from CGD patients.

To increase the transduction efficiency, different strategies have been used to enrich for genecorrected cells. Sokolic et al (377) constructed a bicistronic retrovirus vector containing the X-CGD gene and the human multidrug resistant gene (MDR1) and transduced EBV-cells from a X-CGD patient under vincristine selection. The resulting population of EBV-cells had a similar oxidase activity as normal EBV-cells. Iwata et al (188) used a similar drug selection approach in which a retroviral vector containing the (p47.sup.phox) gene and MDR1 was used to transduce (p47.sup.phox)-deficient EBV-cells, resulting in complete correction of the defect. Becker et al (31) used a retrovirus containing the (gp91.sup.phox) gene to transduce a (gp91.sup.phox)-deficient cell line. Using an optimized gene transfer protocol, which included precoating plates with recombinant fibronectin, up to 85% of (CD34.sup.+) cells obtained from bone marrow of an X-CGD patient were transduced, without positive selection with magnetic beads.

Recently, a recombinant adeno-associated vector containing mouse (gp91.sup.phox) cDNA was used to transduce successfully a myeloid cell line from an X-CGD patient (241). Reconstitution of NADPH oxidase activity and expression of (gp91.sup.phox) persisted for several months without selection. Adenoviral and adeno-associated vectors can transduce nondividing and dividing cells, whereas retroviral vectors only transduce dividing cells (326).

Recently, a clinical trial of gene therapy was conducted at the NIH in 5 patients with the (p47.sup.phox)-deficient form of CGD (254). Autologous (CD34.sup.+) peripheral blood stem cells were transduced in vitro with the retrovirus vector containing (p47.sup.phox) cDNA, and gene-corrected stem cells were infused without prior marrow conditioning. Corrected granulocytes were detectable in the peripheral blood of all patients. As determined by the DHR flow cytometry, peak levels of corrected granulocytes were present at 3-6 weeks after infusion, and ranged from 0.004% to 0.05% of circulating granulocytes. In 2 of 5 patients,

gene-corrected granulocytes were still detected 6 months after infusion.

Summary

The reduced nicotinamide dinucleotide phosphate (NADPH) oxidase complex allows phagocytes to rapidly convert (O.sub.2) to superoxide anion which then generates other antimicrobial reactive oxygen intermediates, such as (H.sub.2)(O.sub.2), hydroxyl anion, and peroxynitrite anion. Chronic granulomatous disease (CGD) results from a defect in any of the 4 subunits of the NADPH oxidase and is characterized by recurrent life-threatening bacterial and fungal infections and abnormal tissue granuloma formation.

Activation of the NADPH oxidase requires translocation of the cytosolic subunits (p47.sup.phox) (phagocyte oxidase), (p67.sup.phox), and the low molecular weight GT-Pase Rac, to the membrane-bound flavocytochrome, a heterodimer composed of the **heavy chain** (gp91.sup.phox) and the light chain (p22.sup.phox). This complex transfers electrons from NADPH on the cytoplasmic side to (O.sub.2) on the vacuolar or extracellular side, thereby generating superoxide anion. Activation of the NADPH oxidase requires complex rearrangements between the protein subunits, which are in part mediated by noncovalent binding between src-homology 3 domains (SH3 domains) and proline-rich motifs.

Outpatient management of CGD patients relies on the use of prophylactic antibiotics and interferon-(Gamma). When infection is suspected, aggressive effort to obtain culture material is required. Treatment of infections involves prolonged use of systemic antibiotics, surgical debridement when feasible, and, in severe infections, use of granulocyte transfusions.

Mouse knockout models of CGD have been created in which to examine aspects of pathophysiology and therapy. Gene therapy and bone marrow transplantation trials in CGD patients are ongoing and show great promise.

(*) This 6-digit number is the entry number in OMIM (Online Mendelian Inheritance in Man), a continuously updated electronic catalog of human genes and genetic disorders. It is authored and edited in the McKusick/Nathans Institute of Genetic Medicine, Johns Hopkins University. The book (259a) and CD-ROM versions of the catalog are published by the Johns Hopkins University Press. The online version is accessible from the National Center for Biotechnology Information, National Library of Medicine through World Wide Web (http://www.ncbi.nlm.nih.gov/omim/).

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Abbreviations used in this article: BMT, bone marrow transplantation; CDP, CCAAT displacement protein; CGD, chronic granulomatous disease; EBV, Epstein-Barr virus; FAD, flavin adenine dinucleotide; INF-(Gamma), interferon-(Gamma); NADPH, reduced nicotinamide dinucleotide phosphate; NBT, nitroblue tetrazolium; NCF-1, neutrophil cytosolic factor-1; NCF-2, neutrophil cytosolic factor 2; PAK, p21-activated kinase; PKC, protein kinase C; PLD, phospholipase D; PMA, phorbol myristate acetate; SH3, src-homology 3 domains; TNF-(Alpha), tumor necrosis factor-(Alpha).

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Botulinum **toxin: from poison to medicine.**

Davis, Larry E.

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AUTHOR ABSTRACT: Although thousands of people in the world each year continue to be poisoned with **botulinum** toxin - food-borne, infantile, or wound botulism - the neurotoxin is now sufficiently understood to allow it to be used as a medicinal agent to paralyze specific muscles, giving temporary symptomatic relief from a variety of dystonic neurologic disorders. I review some of the epidemiologic, clinical, and pathophysiologic aspects of **botulinum** toxin and how the neurotoxin may act as a poison or a medicine.

TEXT:

In the past several years, we have seen the following patients: A 43-year-old man who became flaccidly paralyzed two days after eating his wife's home-canned chilies; a previously healthy 4-month-old baby girl in whom diffuse weakness and constipation developed; a 50-year-old drug addict who subcutaneously inoculated heroin and in whom moderately severe weakness of all limbs and dysphasia developed; and a 45-year-old woman with severe blepharospasm who had pronounced relief of involuntary eyelid closures after her physician injected **botulinum** toxin (Botox) into the orbicularis oculi muscles. All four persons had exposure to the **botulinum** toxin. The first patient had food-borne botulism, the baby had infantile botulism, the heroin addict had "wound" botulism, and the last patient was therapeutically administered **botulinum** A toxin. In this article I review how the **botulinum** toxin can cause these clinical events.

Clostridium **Botulinum**

Clostridium **botulinum** are gram-positive, rod-shaped, spore-forming, strictly anaerobic bacteria that are widely distributed in soil and water.[1] Strains of C **botulinum** produce seven different neurotoxins and one binary toxin. Toxin types A, B, E, and F are the main toxins that affect humans.[1] Toxin types C and D cause botulism in birds and mammals.[2] Clostridium **botulinum** strains that produce toxin types A, B, and F are found generally in soil of geographic areas having low rainfall and moderate temperatures. Type A-producing bacteria are found primarily west of the Mississippi River and type B east of the Mississippi River.[2] Type E-producing bacteria are found in the marine life and sediment around fresh water, especially in Alaska and the Great Lakes region.

Clostridium **botulinum** is a spore-forming bacterium. The spores are capable of surviving 100 [degrees] C for at least six hours, but they are

killed at temperatures of 120 [degrees] C lasting five minutes.[3] The killing temperature of spores is important to the canning industry and was responsible for the practice of canning home foods in a pressure cooker. Home canning done after simply boiling the food is not sufficient to kill the spores. Under anaerobic or semianaerobic conditions, the spores can germinate, with progeny bacteria producing the **botulinum** toxin.[2]

The DNA necessary for C **botulinum** to produce the toxin may be in the host genome or come from either a bacteriophage or a plasmid. Removal of the phage or plasmid from C **botulinum** can result in a nontoxigenic bacterium.[4] Why C **botulinum** normally produces the **botulinal** toxin is unknown as infection of vertebrates is not part of the normal clostridial life cycle, and it has no known role in the growth or physiology of the organism.[5]

Botulinum Toxin

Botulinum toxin is a family of serologically closely related neurotoxins - A, B, [C.sub.1], D, E, F, and G. The primary structure of most serotypes has been determined. The toxins are mainly produced by different strains of clostridia,[2] but six other groups of physiologically distinct clostridia, such as Clostridium barati and Clostridium butyricum, have produced **botulinum** type E and F toxins.[6] On a milligram-per-kilogram basis, they are probably the most potent biologic toxins that affect humans. It has been estimated that 0.025 ng or 2 x [10.sub.7] molecules of the **botulinal** toxin are sufficient to kill a mouse.[7] For comparison, it is estimated that 5 x [10.sup.13] molecules of [alpha]-bungarotoxin (cobra venom) and 2 x [10.sup.18] molecules of cyanide are required to kill a mouse.[8] For humans, about 0.3 ng or [10.sup.11] molecules of **botulinum** toxin reaching neuromuscular junctions are enough to cause clinical botulism.[9]

Botulinum toxin is odorless and tasteless. Unlike C **botulinum** spores, the toxin is heat-labile and is denatured by heating above 80 [degrees] C.[3,10] It can resist destruction by stomach acid and digestion by enzymes in the gastrointestinal tract.

Botulinum toxin shares many properties with the other bacterial toxins such as tetanospasmin and diphtheria toxin.[11] Type A toxin is produced as a single-chain polypeptide with a molecular weight of 150,000.[11] Later it is transformed into its active structure by nicking with a protease to produce a dichain polypeptide of about 100,000 and 50,000 molecular weight.[12] There are three major domains in the molecule (Figure 1): The receptor binding site is at the **carboxyterminus** of the **heavy chain** ([B.sub.3]). The channel-forming domain is the aminoterminus of the **heavy chain** . The internal toxin domain resides in the lighter chain, and the two chains are held together with a disulfide bond.

The pathophysiology of this toxin involves several steps: systemic absorption, binding to the nerve terminal, internalization, and synaptic poisoning. The toxin reaches the lymphatic channels first and then the bloodstream either by absorption through the upper gastrointestinal tract (food-borne and infantile botulism) or through tissue absorption (wound botulism).[9] The toxin then circulates in the blood until it reaches cholinergic synapses in the peripheral nervous system. The toxin appears not to cross the blood-brain barrier so central nervous system cholinergic synapses are not involved.[13] The toxin binds to the presynaptic side of the cholinergic synapse.[11,13] This binding step involves attachment of the **carboxyterminus** of the **heavy chain** of the toxin molecule to receptors on the presynaptic side of the synapse.[14] It has been estimated that there are about 150 to 600 binding sites per micrometer of presynaptic membrane.[8] Binding of the **botulinal** toxin to its receptor is of high affinity. Type-specific **botulinum** antibody is capable of neutralizing the toxin during the period (about 1/2 hour) between binding and internalization.[15] Internalization of the toxin occurs through receptor-mediated endocytosis.[8] In a final step, the **botulinal** toxin light chain crosses the membrane of the endocytic vesicle and enters the cytoplasm of the presynaptic terminal. As the pH in the vesicle falls below 4.0, a conformational change in the toxin molecule occurs. The **heavy chain** enters the vesical membrane to form a channel through which the light chain then passes into the cytoplasm (Figure 2).

On entrance of the light chain into the cytoplasm, the toxin induces dysfunction of the presynaptic terminal. **Botulinum** toxin blocks stimulus-induced and spontaneous quantal acetylcholine release by the presynaptic cholinergic synapse.[11,16] There is evidence that the

inhibitory effect of the toxin occurs selectively at vesical release sites on the membrane of the presynaptic terminal. The blockage of the acetylcholine release results in muscle weakness.

The biochemical basis for the blockade of acetylcholine release is still unknown, but related bacterial toxins provide some clues. For many bacterial toxins, the toxic molecule appears to be an enzyme or regulatory protein. For example, anthrax toxin appears to be an adenylate cyclase.[17] The cholera toxin is an adenosine diphosphate (ADP)-ribosylating regulatory protein that stimulates adenylate cyclase.[18] The pertussis toxin is an ADP-ribosylating regulatory protein that inhibits adenylate cyclase.[18] The **botulinum** [C.sub.2] toxin (not a neurotoxin) appears to be an ADP-ribosyltransferase enzyme.[19] Thus the **botulinal** neurotoxins are likely to be enzymes that amplify inhibitory molecules or regulatory proteins that act to reduce quantal acetylcholine release.[5]

The blockade of quantal acetylcholine release by **botulinal** toxin may last several months. Synaptic acetylcholine transmission may recover through a turnover of critical presynaptic molecules by the cell or by the production of a new synapse through the sprouting of motoneuron axon terminals with the development of an adjacent new synapse on the same muscle fiber.[20,21]

Food-borne Botulism

Food-borne botulism accounts for most of the cases of **botulinal** intoxication. Food-borne botulism occurs when a person eats preformed toxin. Home foods, especially fruits and vegetables that are improperly processed, account for 90% of the cases of botulism.

Most outbreaks of type E botulism involve fish or fish products.[22] In Alaska, botulism among Native Americans has occurred from eating uncooked, dried, or fermented fish.[23]

After toxin ingestion, the mean incubation period is 2 days with a range of 1/2 to 6 days.[24] In general, the longer the incubation period, the milder the signs and symptoms. The classic clinical features of botulism include a descending symmetric paralysis of all skeletal muscles and many smooth muscles.[2,24] Limb muscles become weak or completely paralyzed. Cranial nerve musculature is usually involved, resulting in an external ophthalmoplegia, dysarthria, dysphasia, ptosis, and facial weakness. In addition, patients have a dry mouth and often fixed dilated pupils. Weakness of respiratory muscles may be severe enough to require intubation and mechanical ventilation. Smooth muscle paralysis typically involves the gastrointestinal tract and bladder, resulting in constipation and at times paralytic ileus and urinary retention. Occasionally paresthesias or asymmetric limb weakness may develop.[25] The signs and symptoms of botulism usually progress over one to three days. There are several important negative clinical features of botulism. Because the toxin inactivates cholinergic synapses of the peripheral nervous system, patients usually have normal sensation, normal mentation, normal memory, and normal temperature, blood pressure, and heart rate.[2]

The **botulinum** toxin has been shown in experiments to bind to cholinergic synapses in brain homogenates and to block cholinergic transmission.[5] Patients do not show signs of central nervous system cholinergic blockade, however.[26] The difference may reflect the inability of the toxin to cross the blood-brain barrier or to block completely central nervous system cholinergic transmission.

Electrophysiologic studies are often helpful in the diagnosis of botulism. A modest increment in M-wave amplitude with rapid repetitive stimulation often occurs and may provide an early clue to the cause of the weakness.[27,28] Less specific changes include low-amplitude and short-duration motor unit action potentials with small M-wave amplitudes on an electromyogram. Single-fiber electromyography may show increased jitter and blocking.[27] Motor conduction velocities and distal latencies are normal. Patients with botulism usually have a normal hemogram, urinalysis, and cerebrospinal fluid.

The differential diagnosis of food-borne botulism includes the Guillain-Barre syndrome, diphtheric polyneuropathy, tick paralysis, curare poisoning, poliomyelitis, myasthenia gravis, and the Eaton-Lambert syndrome. If there is a cluster of patients with similar symptoms, botulism should be high in the differential diagnosis.

The laboratory diagnosis of botulism is made by analyzing specimens of serum (20 to 30 ml), stool (25 grams), and suspected food for the **botulinum** toxin.[2] Despite modern serologic tests such as the

radioimmunoassay, the standard and most sensitive diagnostic test for the **botulinum** toxin is a biologic test involving mice. The number of toxin molecules needed to paralyze mouse neuromuscular junctions and to kill mice from respiratory failure is lower than the minimum amount of toxin that currently is needed for detection by standard serologic tests using antibodies against the **botulinum** toxin. The mouse test involves intraperitoneal inoculation of a mouse with the patient's serum or extracts of stool or food specimens to determine whether the mouse becomes paralyzed and dies.[2] Heating the specimen to 100 [degrees] C or combining it with specific **botulinum** antitoxin should prevent the animal's death.

Treatment should begin as soon as the diagnosis of botulism is made. In many situations, a firm clinical diagnosis of botulism can be made even before the laboratory confirms the diagnosis. The treatment of food-borne botulism involves three steps: neutralize the circulating toxin; support the patient; and remove excess toxin, if possible, from the gastrointestinal tract. Because most cases of human botulism belong to types A, B, and E, it is possible to give equine anti-A, B, E serum to patients.[2] One vial (10 ml) should be given intramuscularly and the second vial intravenously. The antitoxin is not available in hospitals or at most state laboratories. In general, the antitoxin must be obtained from regional branches of the Centers for Disease Control (CDC). If botulism is suspected, the physician should contact the botulism officer at the CDC ([1404]) 639-3753, days; or [404] 639-2888, evenings and weekends). At that time, the public health officer can release the antitoxin from the nearest CDC branch to be placed on the next available commercial airline.

Equine **botulinal** antitoxin is extremely potent and is capable of neutralizing 10 to 1,000 times the amount of toxin that would be expected in the blood of most patients.[29] Because the antitoxin is of equine origin, the patient's skin should be tested with antitoxin first. There is about a 4% incidence of subsequent serum sickness.[30]

Botulinal antitoxin will not reverse existing clinical weakness as the toxin has already been internalized and is not accessible to antibody neutralization. Nevertheless, there does appear to be value in neutralizing any toxin that is still in the circulation. In one study, patients receiving the antitoxin on day 1 of their symptoms had fewer days on a respirator and shorter hospital stays than those receiving antitoxin after the first day.[31] To date, patients with botulism have not had a notable improvement from the administration of drugs that increase the release of acetylcholine from normal cholinergic synapses (guanidine, or 3,4-diaminopyridine) or that inhibit the breakdown of acetylcholine in the synaptic cleft (pyridostigmine).[32,33]

Because weakness may progress for several days, it is important to place patients in intensive care and monitor them carefully. Should respirations begin to fail, intubation and assisted ventilation are required. The respiratory weakness is often prolonged, necessitating a tracheostomy. Patients may have an ileus for several days, so feeding through a nasogastric tube should not begin until bowel sounds have returned. Frequently the bladder paralysis necessitates the use of an indwelling urinary catheter. It would be ideal to remove unabsorbed toxin from the gastrointestinal tract by the use of laxatives or enemas, but if gastrointestinal tract paralysis has occurred, this should not be undertaken.

Recovery from botulism is slow. Patients may be in the hospital for as long as six months and may be on a respirator for weeks to a few months. Even after hospital discharge, patients often complain of fatigue or dyspnea on exertion for at least a year. Muscle strength tests and pulmonary function tests are usually normal, however.[34] Patients who recover from botulism do not have antibodies to **botulinum** toxin, since the intoxication results from insufficient **antigen** to stimulate the immune system. The mortality from food-borne botulism has decreased in the United States primarily because of better intensive care. From 1900 to 1950 about 60% of patients died, whereas from 1970 to 1975 only 21% of persons died of food-borne botulism.[35]

Infantile Botulism

Infantile botulism results from the ingestion of clostridial spores that then colonize the gut to produce their neurotoxin directly in the gut.[36] The normal gastrointestinal tract of children and adults is remarkably resistant to colonization by clostridial bacteria. The gastrointestinal tract of infants I week to 11 months of age may, however,

occasionally allow colonization of this organism. Factors influencing the vulnerability of the infant gut to colonization are poorly understood, but some infections occur when breast-feeding is changed to bottle-feeding. Under rare circumstances, adults have suffered a similar colonization of the gastrointestinal tract by clostridial organisms. In general, these adults have had their gut flora and pH altered by a surgical procedure or drugs.[37]

The average age of patients with infantile botulism is 3 months, but cases occur in infants 1 to 11 months.[38,39] Infants may have mild or severe disease.[40] Constipation from a partially paralyzed ileus usually develops first and can persist as long as three weeks before other symptoms appear.[38,41] A flaccid paralysis then develops, and the infants have a weak cry, poor suck, and a decreased interest in their environment. Loss of head control is a common early sign. Cranial nerve palsies are common, often producing ptosis, dilated pupils, facial diplegia, and impaired gag reflex.[38] Decreased respirations may develop, and respiratory assistance may be required. Deep tendon reflexes are decreased or absent. Autonomic involvement usually includes dry mucous membranes, decreased bowel motility, and urinary retention. The children are usually afebrile.

The differential diagnosis includes dehydration or electrolyte imbalance, diphtheric polyneuropathy, neonatal myasthenia gravis, poliomyelitis, hypothyroidism, tick paralysis, Werdning-Hoffmann spinal muscular atrophy, Leigh disease (subacute necrotizing encephalomyelopathy), congenital myopathy, the Guillain-Barre syndrome, and exposure to toxins such as heavy metals and organophosphates.[42]

Similar to those with food-borne botulism, patients with infantile botulism usually have reduced amplitude of their evoked compound muscle action potentials and incremental responses following repetitive nerve stimulation.

The diagnosis of infantile botulism depends on the recovery of clostridial organisms and toxin from the stool.[2,44] Toxin and organisms may persist in the stool for a long time, even after clinical recovery.[45] Toxin is seldom detected in serum.[45]

The treatment of infantile botulism differs from food-borne botulism. **Botulinal** antitoxin has not affected the outcome of infantile botulism and is generally not given.[46] Similarly, penicillin or other antibiotic therapy has not affected the outcome of the disease.[38,47] Aminoglycoside antibiotics are contraindicated because they increase presynaptic muscular blockade and may worsen the paralysis.[48] Therefore, treatment is excellent supportive care in an intensive care unit. Because the infant stool contains viable clostridia, care should be taken not to cross-infect other infants in the intensive care unit. Infants usually recover spontaneously over several weeks. Currently less than 3% of patients in hospital die.[46]

The age distribution of the sudden infant death syndrome parallels that of infantile botulism.[40] Studies of infants dying of the sudden infant death syndrome have shown that about 5% have evidence of intestinal infection with C **botulinum** , suggesting that infantile botulism may be one cause of the syndrome.[40,41] It should be noted that C **botulinum** organisms have also been identified in healthy infants without signs of botulism.

Wound Botulism

Wound botulism is the least common type of **botulinal** intoxication. Wound botulism occurs when C **botulinum** replicates in an abscess that has an anaerobic environment producing **botulinal** toxin that becomes systemically absorbed. Wound botulism is a rare disease in the United States and occurs most often from subcutaneous abscesses or deeper wound infections in muscle, nerves, and soft tissues.[41] At present, most cases occur in drug abusers who subcutaneously inject street drugs ("skin popping")[50] or following penetrating trauma where the wound becomes infected.[51] The incubation period from infected wounds varies from 4 to 17 days.[51]

Signs and symptoms consistent with botulism generally develop over several days. Electrodiagnostic study findings are usually similar to those seen in food-borne botulism.[50]

The diagnosis of wound botulism is best made by the isolation of C **botulinum** organisms from the infected abscess. The infected wound should be searched for with care because it may not be obvious. Irrigating the abscess with a sterile saline solution and subsequent culture of the

recovered fluid in anaerobic culture media may yield the organism. Unfortunately, the toxin often is not detected in serum, and in wound botulism, the toxin is never found in stool.

The treatment of wound botulism includes debridement of the abscess or infected wound. Penicillin should be administered along with the **botulinum** antitoxin. Patients should be placed in intensive care and given supportive care, including endotracheal intubation and mechanical ventilation if necessary.

Botulinum A Toxin as a Medication

As the mechanism of action of the **botulinum** toxin became better understood, it was recognized that the toxin could be used selectively to paralyze muscles.[52] Purified type A **botulinum** toxin is now used to treat various neurologic disorders including blepharospasm, spasmodic torticollis, hemifacial spasm, strabismus, and laryngeal dystonia or spastic dysphonia.[53-55] The dystonic or spasmodic neurologic illnesses are characterized by involuntary intermittent or sustained contractures of specific groups of muscles. They have been difficult to treat with other medications because the dosage required for any symptomatic relief usually weakens all muscles.

The administration of minute amounts of **botulinal** toxin into specific skeletal muscles causes localized muscle weakness or paralysis by blocking acetylcholine release at the neuromuscular junctions of only the treated muscles. In addition, there is a diminution of the dystonia or tonic muscle spasms. **Botulinal** toxin treatment of these facial or neck muscle disorders has given dramatic, albeit temporary, symptomatic relief to thousands of patients. **Botulinal** toxin treatment usually gives relief for 4 to 12 months before the condition returns. At that time, the **botulinal** toxin is often readministered to the same muscles. Repeated muscle injections may provide relief for shorter periods than the initial administration. It is not yet clear if **botulinal** toxin can be readministered indefinitely or whether the effectiveness eventually wears off.

Care must be taken when the toxin is administered because it can diffuse from the inoculation site through tissue to paralyze neuromuscular junctions of adjacent muscles, causing unwanted side effects such as muscle weakness and dysphagia. In addition, occasionally antibodies against the toxin have developed in patients receiving large doses of the toxin, and these patients do not benefit from repeated toxin injections.

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SPECIAL FEATURES: illustration; chart
DESCRIPTORS: **Botulinum** toxin--Therapeutic use; Neurotoxic agents--Physiological aspects
FILE SEGMENT: HI File 149

6/9/20 (Item 1 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

13559132 PMID: 9246568

Localization of the regions on the C - terminal **domain of the** heavy chain **of** botulinum **A recognized by T lymphocytes and by antibodies after immunization of mice with pentavalent toxoid.**

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We have **mapped** the regions recognized by T and/or B cells (Abs) on the **C - terminal** domain (Hc) of the **heavy chain** of **botulinum** neurotoxin serotype A (BoNT/A) after immunization of two inbred mouse strains with pentavalent toxoid (BoNTs A, B, C, D and E). Using a set of synthetic overlapping peptides, encompassing the entire Hc domain (residues 855-1296), we demonstrated that T cells of Balb/c (H-2d) mice, primed with one injection of toxoid, recognized two major regions within residues 897-915 and 939-957. After multiple inoculations with toxoid, T cells of Balb/c expanded their recognition ability and responded very well to challenge with peptide 1261-1279 and moderately to stimulation with peptide 1149-1167. Unlike Balb/c T cells, those of toxoid-primed SJL (H-2s) mice exhibited a more complex profile and responded to challenge with a large number of overlapping peptides. After one toxoid injection, however, three peptides, 897-915, 939-957/953-971 overlap and 1051-1069, were the most potent T cells stimulators. After three toxoid injections, peptides 897-915 and 1051-1069 remained immunodominant while the third region was shifted upstream to 925-943/939-957 overlap. The immunodominant **epitope** within peptide 897-915 was recognized exclusively by T cells, since no Abs were detected against this region. The Ab binding profiles of the two mouse strains were quite similar, showing only small quantitative differences. Both, Balb/c and SJL anti-toxoid Abs displayed strong binding mainly to peptide 1177-1195, followed by peptides 869-887/883-901 overlap and 1275-1296. In addition, a significant amount of Balb/c anti-toxoid Abs was bound to peptide 1135-1153. Unlike Balb/c Abs, that interacted weakly with peptides 995-1013 and 1051-1069, the anti-toxoid Abs of SJL mice exhibited strong binding toward both peptides. The results showed that, in a given strain, the regions recognized by anti-toxoid Abs and T cells may coincide or may be uniquely B or T cell determinants.

Tags: Female; Support, Non-U.S. Gov't; Support, U.S. Gov't, Non-P.H.S.
Descriptors: Antibodies, Bacterial; * **Botulinum** Toxin Type A--chemistry --CH; * **Botulinum** Toxin Type A--immunology--IM; *T-Lymphocytes --immunology--IM; Amino Acid Sequence; Animals; Bacterial Vaccines --isolation and purification--IP; **Botulinum** Toxin Type A--genetics--GE; Clostridium **botulinum** --immunology--IM; **Epitope Mapping** ; Immunization; Lymphocyte Activation; Mice; Mice, Inbred BALB C; Molecular Sequence Data; Peptide Fragments--chemistry--CH; Peptide Fragments--genetics--GE;

Peptide Fragments--immunology--IM; Toxoids--administration and dosage--AD
CAS Registry No.: 0 (Antibodies, Bacterial); 0 (Bacterial Vaccines); 0 (Botulinum Toxin Type A); 0 (Clostridium botulinum toxoid); 0 (Peptide Fragments); 0 (Toxoids)
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00313313
IDENTIFYING NO.: 1R21AI55946-01 AGENCY CODE: CRISP
New genetic vaccine to protect aganist botulism
PRINCIPAL INVESTIGATOR: ZENG, MINGTAO
ADDRESS: MINGTAO ZENG@URMC.ROCHESTER.EDU UNIV OF ROCHESTER MEDICAL CTR 601 ELMWOOD AVENUE, BOX 672
PERFORMING ORG.: UNIVERSITY OF ROCHESTER, ROCHESTER, NEW YORK
SPONSORING ORG.: NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES
DATES: 2008/01/03 TO 2007/31/05 FY : 2003 TYPE OF AWARD: New Award (Type 1)
SUMMARY: DESCRIPTION (provided by applicant): Botulism is a severe neuroparalytic disease caused by one of seven **botulinum** neurotoxins (BoNTs), produced by the anaerobic, spore-forming bacterium Clostridium **botulinum** . These protein neurotoxins are the most potent toxins known to man. There are BoNT toxoid vaccines available currently as Investigational New Drugs. However, due to the numerous shortcomings associated with the toxoid vaccines (i.e., dangerous to produce, high cost of manufacturing, high reactogenicity), there is an urgent need to develop new generation vaccines for the prevention of botulism. The goals of this research are to develop a new botulism vaccine using the **carboxyl - terminal** 50 kDa **C** -fragments (Hc) of the **heavy chains** in BoNTs as **antigens** and to study the delivery of the vaccine utilizing a replication-defective adenoviral vector via the intranasal and transcutaneous routes. These non-invasive vaccine delivery methods will undoubtedly enhance the compliance of a vaccination program, which is especially critical in response to a potential bioterrorist attack using BoNTs. After construction of replication-defective adenoviral vectors encoding the immunogenic C-fragments of the **heavy chains** in BoNTs, vaccination protocols in mice comparing the intranasal and transcutaneous delivery modes with the subcutaneous injection of the currently available pentavalent **botulinum** toxoid vaccine (PBT) will be studied. The specific aims of this project are: Specific Aim #1: To construct replication-defective adenoviral vectors encoding the C-fragments of the **heavy chains** in BoNTs. Specific Aim #2: To study the mucosal and systemic immunity elicited by the vectored vaccine developed in aim #1 through intranasal and transcutaneous immunization in a mouse model.
DESCRIPTORS: laboratory mouse; polymerase chain reaction; inhalation drug administration; topical drug application; colony stimulating factor; active immunization; toxoid; enzyme linked immunosorbent assay; western blotting; antiviral antibody; **botulinum** toxin; neurotoxin; tissue /cell culture; Adenoviridae; neutralizing antibody; mucosal immunity; vaccine development; vector vaccine; botulism; Clostridium **botulinum**

6/9/22 (Item 1 from file: 399)
DIALOG(R)File 399:CA SEARCH(R)

128256129 CA: 128(21)256129q JOURNAL
Immune recognition of botulinum neurotoxin type A: regions recognized by T cells and antibodies against the protective Hc fragment (residues 855-1296) of the toxin
AUTHOR(S): Oshima, Minako; Hayakari, Makoto; Middlebrook, John L.; Atassi, M. Zouhair
LOCATION: Dep. Biochem., Baylor College Medicine, Houston, TX, 77030, USA
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IDENTIFIERS: Clostridium neurotoxin epitope T cell, antibody epitope
Clostridium neurotoxin
DESCRIPTORS:
Antibodies... Epitopes... T cell(lymphocyte)...
epitope mapping of botulinum neurotoxin A heavy chain C-terminus
CAS REGISTRY NUMBERS:
93384-43-1 194663-25-7 194663-27-9 194663-29-1 194663-31-5 194663-33-7
194663-34-8 194663-36-0 205234-84-0 205234-85-1 205234-86-2
205234-87-3 205234-88-4 205234-89-5 205234-90-8 205234-91-9
205234-92-0 205234-93-1 205234-94-2 epitope mapping of botulinum
neurotoxin A heavy chain C-terminus
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           $0.01    0.002 DialUnits File369
    $0.01  Estimated cost File369
           $0.01    0.002 DialUnits File370
    $0.01  Estimated cost File370
           $0.02    0.002 DialUnits File399
              $2.75  1 Type(s) in Format  9
           $2.75  1 Types
    $2.77  Estimated cost File399
           $0.03    0.002 DialUnits File434
    $0.03  Estimated cost File434
           $0.01    0.002 DialUnits File444
    $0.01  Estimated cost File444
           $0.01    0.002 DialUnits File467
    $0.01  Estimated cost File467
           OneSearch, 26 files,  2.787 DialUnits FileOS
    $0.50  TELNET
   $73.80  Estimated cost this search
   $73.80  Estimated total session cost   2.787 DialUnits


### Status: Signed Off. (2 minutes)
```